THE EFFECT OF SULFATE DEPLETING DRUGS ON CARTILAGE METABOLISM

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CONTENTS

CHAPTER 1

INTRODUCTION

EPIDEMIOLOGY OF OSTEOARTHRITIS

Osteoarthritis (osteoarthrosis, degenerative joint disease) is the most frequent joint disorder among the human population. It involves any population, independent of geographic localization or climate (1). Comparison of osteoarthritis prevalence in 17 populations (white, black or indian) from Alaska to South Africa demonstrated no statistically significant difference in overall prevalence (1). However, other radiographic studies among different populations showed that American indians have a significantly increased prevalence of osteoarthritis (2) while eskimos have a significantly lower incidence of osteoarthritis than found in United States males and females (3).

The prevalence of osteoarthritis demonstrates a high correlation with age. In a nationwide study in the United States of America all degrees of radiographic osteoarthritis of the extremities increased with age from 4% in the age group 18 to 24 years to 85% among individuals 75 to 79 years of age (2). In the Framington osteoarthritis study, radiographic evidence of knee joint osteoarthritis increased with age from 27% in individuals younger than 70, to 44% in individuals age 80 or older (4). Males appear to be affected more commonly than females before 50 years of age, but the sex ratio is reversed thereafter (5). In the Framington Study cited above similar observations were made, the age associated increase in osteoarthritis was almost completely the result of the increase in the incidence of osteoarthritis among the women studied (4).

Epidemiological studies have suggested a role for mechanical factors in the pathogenesis of osteoarthritis but the overall picture is not clear yet. Osteoarthritis shows significant differences by occupation and industry. Office workers had a lower than expected frequency of hand involvement but higher than expected foot involvement (6). The opposite was observed in craftsmen, foremen and similar workers (6). In a cross sectional epidemiologic study in a urban population the incidence of osteoarthritis was higher in the right hand than

in the left hand suggesting a role for usage in the pathogenesis (7). Moreover, the pattern of hand usage among industrial workers showed significant task-related osteoarthritic changes in the hands (8). Osteoarthritis of elbow, wrist and shoulder and hands has been reported in users of pneumatic drills (9,10,11,12). However, competitive sports like running or American football, were not found to predispose to osteoarthritis of the hips or ankles (13,14).

PATHOGENESIS OF OSTEOARTHRITIS

Osteoarthritis is a joint disease with a multifactorial etiopathogenesis in which the prominent feature is degeneration of articular cartilage. Osteoarthritis can be largely divided in two groups; primary or "idiopathic osteoarthritis" with unidentified etiologic factors and secondary osteoarthritis with known pathogenetic factors. The various forms of osteoarthritis can be considered as being the result of either abnormal mechanical stress or congenital and acquired aberrations of articular cartilage, or a combination of both. Regardless of the pathogenetic pathway, the final outcome is disturbed cartilage integrity.

Articular cartilage

Articular cartilage is a specialized form of connective tissue consisting of cartilage cells (chondrocytes, approximately 10% total volume) which synthesize and deposit around themselves an extracellular matrix of giant molecules. These macromolecules are responsible for the biomechanical and biochemical properties of articular cartilage making this tissue unique in its function as shock absorber and gliding surface for the diarthrodial joint. The major macromolecules deposited by the chondrocytes in the extracellular matrix are collagen type II and proteoglycans. The collagen type II provides the tensile strength of cartilage while the proteoglycans are responsible for the resiliency and water binding capacity of this tissue.
A proteoglycan is "any molecule that has a core protein containing at least one covalently bound glycosaminoglycan

chain" (15). A glycosaminoglycan is a linear polymer consisting of repeating disaccharides containing hexosamine and hexuronic acid or hexose. The chains are highly negatively charged due to the carboxyl residues and/or sulfate ester groups attached to the disaccharides. The negative charges on the glycosaminoglycans are essential for the pressure resistent properties and resiliency of the cartilage proteoglycans.

The major cartilage proteoglycans are high-molecular-weight molecules up to 3 x 10^6 consisting of a large number of glycosaminoglycans covalently linked to the central core protein (16). The carbohydrate component can contribute 90% of the total weight of the molecule. These cartilage proteoglycans may contain up to 100 chondroitin sulfate chains, up to 50 keratan sulfate chains and up to 50 O-linked and 15 N-linked oligosaccharide chains (17,18,19,20,21). The large cartilage proteoglycan of mice and rats has been reported to contain no keratan sulfate (22,23).

Chondroitin sulfate is the most abundant glycosaminoglycan of articular cartilage and consists of N-acetylgalactosamine and glucuronic acid disaccharides. The disaccharides are sulfated at either the C-4 or C-6 position of the galactosamine and are than termed chondroitin-4-sulfate and chondroitin-6-sulfate. However, no chondroitin sulfate chain is solely sulfated at either the six or the 4 position but will be a co-polymer (24). The above described large cartilage proteoglycan, called proteoglycan monomer, interacts with another glycosaminoglycan, hyaluronic acid, by a globular domain at one end of the core protein, the hyaluronic acid binding site, forming in this way aggregates with a molecular weight over 10^8 (25,26,27,28). The proteoglycan monomer-hyaluronic acid binding is stabilized by interaction with a glycoprotein, called link protein (29,30). The proteoglycan aggregates are due to their large size immobilized within the fibrous collagen network of articular cartilage (31).

Alterations in osteoarthritic joints

The changes occurring in the articular cartilage, as a result of the osteoarthritic process, are manyfold. In both human and

experimental osteoarthritis, one of the first events observed in osteoarthritic cartilage is an elevated hydration of the extracellular matrix and an increased extractability of proteoglycans (32-36). The increased extractability can be due to a weaker binding of proteoglycans within the cartilage matrix framework as a result of changes in the collagen component, or to changes in the proteoglycan structure in osteoarthritic cartilage. Alterations in structure of proteoglycans from human osteoarthritic cartilage and in cartilage from animals with experimental osteoarthritis have been reported frequently.

In experimental knee osteoarthritis in dogs, changes in proteoglycan structure were detected before histologically detectable osteoarthritic alterations occurred (32,37-39). An increased galactosamine to glucosamine ratio, indicating an elevated proportion of chondroitin sulfate to keratan sulfate, was found in canine osteoarthritic cartilage proteoglycans (37-40). In the same model, Altman et al. reported that the proportion of proteoglycans involved in the hyaluronic acid-proteoglycan aggregates was reduced but that there was no detectable overall reduction in proteoglycan monomer size (40). Similar results, a diminished fraction of proteoglycans in aggregates although the extracted proteoglycans appeared to have a functional hyaluronic acid binding site, were reported by McDevitt and Muir (41,42). The reduced proportion of proteoglycans in the proteoglycan hyaluronic acid aggregates might be the consequence of hyaluronic acid depletion in the osteoarthritic cartilage of dogs (43). In the osteoarthritic hip cartilage of dogs with hip dysplasia, the proteoglycan monomers in the degenerated cartilage were smaller than those from normal cartilage of the same age, as a result of a smaller chondroitin sulfate-rich region (44). Also some of the molecules lacked the hyaluronic acid-binding region (44).

In an experimentally induced model of rabbit knee osteoarthritis, no proteoglycan aggregates could be extracted from osteoarthritic cartilage notwithstanding the observation that the proteoglycan monomers had the normal size (33). Also no alterations in proteoglycan structure were observed in osteoarthritic cartilage of STR/IN mice and guinea pigs (34,36).

The changes in proteoglycan structure found in human osteoarthritic cartilage are controversial. Vasan reported a reduction in size of proteoglycan monomers, a reduced chain length of glycosaminoglycans and a diminished ability of proteoglycans to form aggregates with hyaluronic acid in osteoarthritic cartilage from human femoral heads (45). However, Santer and associates were unable to detect changes in aggregate formation, structure and glycosaminoglycan chain length of proteoglycans from osteoarthritic cartilage of the human tibial plateau (46).

The variation in reported proteoglycan changes in osteoarthritic cartilage might be the result of differences in cartilage sampling or differences in the stage of the osteoarthritic process. Variation in the anatomical site of cartilage sampling or differences in the depth of cartilage sampling can lead to dissimilar results in relation to changes in the proteoglycan structure. Also the sampling of the control cartilage might induce variation in conclusions. Sampling of control cartilage from normal donors or sampling of control cartilage from the diseased joint at apparently unaffected anatomical sites might explain part of the variation observed. Moreover, the stage of progression of the osteoarthritic process can interfere with the changes in proteoglycan structure found by different authors.

Besides qualitative changes in proteoglycan structure, obvious quantitative changes in proteoglycans from osteoarthritic cartilage are found. Both in human and experimental osteoarthritis, most authors report a diminished concentration of proteoglycans in osteoarthritic cartilage compared to normal cartilage (39,47-54). This is proposed to be due to breakdown of cartilage proteoglycans by proteases excreted by chondrocytes (55-61). In this viewpoint, osteoarthritis is an autodestructive process, mediated by articular cartilage chondrocytes, triggered by as yet unknown mechanisms (59). The low grade inflammation of osteoarthritic joints, as reported by several authors, can play an additional role in the destructive process by the release of inflammatory mediators such as interleukin 1 (54,61-70). The synovial inflammation in osteoarthritic joints is probably a secondary reaction on the initial event of cartilage destruction while in patients with

rheumatoid arthritis the synovial inflammation in the joints is the primary phenomenon followed by cartilage destruction. Although the concentration of proteoglycans is decreased in osteoarthritic cartilage the synthesis of proteoglycans appears to be elevated in the same cartilage, both in humans and animals (34,47,48,71,72). The proteoglycan synthesis shows a positive correlation with the severity of the osteoarthritic lesions up to a certain degree of disease activity, as shown by Mankin et al. (47,48). Notwithstanding this observation, the proteoglycan concentration in osteoarthritic cartilage is reduced. This indicates that the proteoglycan catabolism is even more elevated than the proteoglycan synthesis in osteoarthritic cartilage (73). Brocklehurst et al. observed no differences in the rate of proteoglycan synthesis or in proteoglycan concentration in cartilage from human osteoarthritic or control knees (35).

Probably in reaction to the decreased proteoglycan concentration in the extracellular matrix, normally non-proliferating articular cartilage chondrocytes are stimulated to divide and as a result of this chondrocyte cell clusters are found in osteoarthritic cartilage (35,38,47,49,50,52,74-76). The proliferation of the chondrocytes and the elevated proteoglycan synthesis can be considered as a reparative response in an attempt to repair the damaged cartilage matrix. Besides changes in the articular cartilage, also other joint structures undergo alterations in the osteoarthritic joints. Osteoarthritic changes in the articular cartilage are associated with osteophyte formation at the joint margins (38,49,50,52,74-77). Another phenomenon seen in osteoarthritic joints is sclerosis of subchondral bone below the degenerated cartilage (53,75,78-80). Chondrocyte proliferation, increased proteoglycan synthesis, osteophyte formation and sclerosis of subchondral bone indicate the presence of anabolic processes in osteoarthritic cartilage in addition to the catabolic degeneration of the cartilage matrix.

ANIMAL MODELS OF OSTEOARTHRITIS

Since the pathogenesis of osteoarthritis can not adequately be studied in humans, animal models which simulate the human

disease have been developed. Spontaneous osteoarthritis has been studied in mice, guinea pigs and dogs (36,44,53,78,80-87). In addition to the spontaneous models, osteoarthritis has been induced in animal joints with essentially two different techniques. First, induction of joint instability by surgical methods and second, interference with cartilage metabolism by intraarticular injection of chemicals.

Induction of knee joint instability has been carried out by dissection of cruciate ligaments (38,39,43,72,76,88-90) and tibial valgus osteotomy (91) in dogs. In rabbits, joint instability was surgically induced in the knee joints by partial meniscectomy, sometimes in combination with collateral ligament dissection, leading to osteoarthritic lesions within several weeks (33,49,50,75). A combination of meniscectomy, dissection of collateral ligaments and cruciate ligaments was used by Hulth et al. (92) and Lane et al. (93) to induce osteoarthritis in rabbit knees. Partial meniscectomy, alone (52) or in combination with collateral and cruciate ligament dissection (34,94), and myotomy and tendotomy (95) was carried out to produce joint instability and subsequently osteoarthritis in guinea pigs. In rats, cruciate ligament dissection was used to induce osteoarthritic knee joint lesions (96).

Papain, iodo-acetate and several drugs have been used to induce osteoarthritic alterations in joints of laboratory animals. Multiple intraarticular injections with papain have been carried out to produce osteoarthritis in the knee and hip joints of rabbits and guinea pigs (97-106). Sodium iodo-acetate, an inhibitor of cell metabolism, has been injected in the knee joints of rats, chicken and guinea pigs resulting in osteoarthritic changes in the injected joints (107-111). Also the injection of certain nonsteroidal antiinflammatory drugs into the knee joint cavity of hens and rats leads to osteoarthritic alterations (107,112,113).

In chapter 7 and 8 the development of three osteoarthritis models in mice is described. A single intraarticular injection of papain or iodo-acetate in the knee joints of the mice leads to osteoarthritic alterations in the joints by way of interfering with the cartilage metabolism. Injection of collagenase results by way of damaging joint structures, such

as ligaments and tendons, to a instable knee joint and eventually to osteoarthritis. This model seems to be comparable to the surgically-induced osteoarthritis in larger animals as described above. The diversity of osteoarthritis models in laboratory animals demonstrate that osteoarthritis can be the pathological result of various etiological factors and most researchers agree that also human osteoarthritis is not a single disease entity but the final outcome of numerous etiologic stimuli (114).

EFFECT OF ANTI-OSTEOARTHRITIC DRUGS ON CARTILAGE METABOLISM

The final outcome of all variants of osteoarthritis is the destruction of articular cartilage leading to the loss of functionality of diarthrodial joints and in this way to disability of osteoarthritic patients. One of the main effects of anti-osteoarthritic drugs should be aimed at the protection of articular cartilage. These drugs should at least have no harmful effect on cartilage metabolism. However, several nonsteroidal antiinflammatory drugs (NSAIDs) used in the treatment of osteoarthritis have been reported to interfere negatively with articular cartilage metabolism (115-134).
Drugs can exert their pharmacological effects on cartilage metabolism in two ways: a direct effect on the biochemical activities of chondrocytes and/or an indirect interference with chondrocyte metabolism by influencing systemic factors determining this metabolism.
Various direct effects Of NSAIDs on chondrocyte metabolism are reported in the literature. Brandt and coworkers have made extensive study of the inhibitory effects of salicylate on glycosaminoglycan synthesis (121-124,126-129). They showed that the salicylate-mediated inhibition was a direct effect on chondrocyte metabolism (124). Also other NSAIDs, such as salicylamide, phenylbutazone, oxyphenbutazone, Ibuprofen, tolmetin, naproxen, fenoprofen, indomethacine, isoxicam, mefenamic acid and sulindac sulfoxide, have been reported to decrease glycosaminoglycan metabolism by a direct mechanism (115,117,118,122,127,131,135).
An indirect effect of salicylate on glycosaminoglycan synthesis in articular cartilage has been demonstrated in

experiments performed in our laboratory (136,137). A decrease of inorganic serum sulfate concentration in mice by way of salicylate-induced sulfate diuresis, apparently was the causative factor generating a reduced rate of sulfate incorporation in anatomically intact patellar cartilage (136,137). The reduced incorporation of sulfate can be caused by a diminished production of normal glycosaminoglycan chains or by the synthesis of a normal quantity but undersulfated glycosaminoglycan chains, or a combination of both. Either a diminished synthesis of glycosaminoglycans or the synthesis of undersulfated glycosaminoglycans will result in cartilage with inferior biomechanical and biochemical properties. Inhibition of cartilage metabolism by direct or indirect mechanisms might accelerate the cartilage destructive process in osteoarthritis patients.

AIM OF THE STUDY

The aim of the present study was to elucidate the sulfate depleting potential of antirheumatic drugs and the effect of reduced sulfate availability on glycosaminoglycan synthesis in articular cartilage. In addition to the above mentioned study, osteoarthritis models in mice, necessary for the future study of anti-osteoarthritic drugs in vivo, have been developed.
In chapter 2 the potential sulfate depleting effects of several anti-rheumatic drugs is evaluated. The effects of limited sulfate availability in vitro on the rate of glycosaminoglycan synthesis and the structure of the glycosaminoglycan chains synthesized are described in chapter 3 and 4. In vivo interference with glycosaminoglycan synthesis by paracetamol-induced sulfate depletion in Wistar rats is described in chapter 5 and 6. Chapter 7 and 8 depict the three newly developed murine osteoarthritis models. Chapter 9 describes the susceptibility of glycosaminoglycan synthesis in articular cartilage of five different species and the different vulnerability of normal and diseased articular cartilage to changes in sulfate availability.

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CHAPTER 2

DECREASE OF INORGANIC BLOOD SULFATE FOLLOWING TREATMENT WITH SELECTED ANTIRHEUMATIC DRUGS: POTENTIAL CONSEQUENCE FOR ARTICULAR CARTILAGE

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Agents and Actions: in press

ABSTRACT

The elimination kinetics of inorganic blood sulfate in mice was followed for four hours after a single, oral administration of an antirheumatic drug. Sodium salicylate, aspirin, diflunisal and benorylate, all in a dose of 1.25 mmol/kg, reduced the sulfate level to less than half that of control. This phenomenon was also demonstrated by phenylbutazone, oxyphenbutazone (both 1 mmol/kg), chloroquine diphosphate (0.6 mmol/kg) and tiaprofenic acid (0.02 - 0.35 mmol/kg). Niflumic acid (1.08 mmol/kg), piroxicam (0.03 mmol/kg), indomethacin ($6. 10^{-3}$ mmol/kg), diclofenac ($5. 10^{-3}$ mmol/kg), ketoprofen (0.2 mmol/kg), naproxen (0.08 mmol/kg) and ibuprofen (0.24 mmol/kg) possessed no sulfate lowering properties. The potential relevance of the use of sulfate lowering drugs for articular cartilage integrity is discussed in the light of what is already known about this subject.

INTRODUCTION

Many phenolic agents are made hydrophilic by conjugation with inorganic sulfate to facilitate elimination out of the body. Some of these are well known drugs like salicylamide and paracetamol. In man, both drugs cause a decrease in circulating inorganic sulfate (1,2). In mice, sodium salicylate decreases the level of inorganic sulfate by another process, i.e. sulfate diuresis (3), which is also the cause of the same phenomenon seen in salicylate treated rats (4,5). A potential consequence of a reduced sulfate availability may be a suppression of biosynthesis of the highly sulfated proteoglycans, abundantly present in fetal skeletal tissue and cartilage, as was shown in mice and rats (3,6,7), leading ultimately to inferior tissue properties.

Unrepairable damage to articular cartilage is an important factor in the disease process by which rheumatic patients, suffering from osteoarthritis or inflammatory arthritis, are becoming disabled. It would probably be a bad choice to prescribe them antirheumatic drugs which have an adverse effect on cartilage. Many studies are documented on the effects of drugs on cartilage (8,9,10,11). So far little attention has been paid to indirect adverse effects, mediated by drug induced disturbance in the supply of essential nutrients needed by cartilage cells, like e.g. inorganic sulfate. Here we report the influence of several, currently used antirheumatic drugs on the inorganic sulfate content in blood of mice.

MATERIALS AND METHODS

Healthy C57Bl10 male mice, aged 8-12 weeks, were injected intraperitoneally with carrier-free $Na_2{}^{35}SO_4$ (0.2 μCi/g), dissolved in physiologic saline. Thirty minutes later, after the maximum concentration of ^{35}S was attained in the blood (12), they received by oral gavage 200 μl of a 5 min ultrasonically trilled drug suspension or solution in 0.1 % methylcellulose or this vehicle alone (controls). At 30, 90 and 240 minutes after drug administration, 10 μl blood samples were taken from the tail vein and suspended in 500 μl distilled water. These samples were subse-

quently analysed by beta liquid scintillation spectrometry (3) for ^{35}S content, which gives an estimate of relative endogenous, inorganic sulfate concentrations in the circulation. Since data from preliminary experiments demonstrated that more than 90% of ^{35}S in serum, derived from normal and drug-treated mice, was associated with inorganic sulfate, as checked by our benzidine method (13), this procedure appeared to be rapid and reliable in our animal model for monitoring endogenous, inorganic sulfate concentrations.
Pure substance-drugs were obtained from several pharmaceutical companies or purchased from Sigma (St Louis, MO).

STATISTICAL ANALYSIS

We used the unpaired, two-tailed Student's t-test to determine the significance of drug induced effects. In each separate experiment a control group of 5 mice was run parallel with drug treated mice (n=5). Due to slight variations in the experimental conditions of the several separate experiments, comparison of data obtained from these separate experiments appeared not surveyable without normalizing the mean values. To this end values of ^{35}S content in blood, originally expressed as cpm's, were converted to radioactivity units. Mean control values were always converted to 100 ± SD units; values from drug-treated mice were adapted accordingly. To give an idea of the deviations in the outcomes of control experiments, the average standard deviation (SD), belonging to the control values, was calculated from 24 separate experiments, see table 1. For each drug tested, statistics was applied with reference to the original control ± SD value. Statistical probability (P) values are mentioned explicitly if $P \leq 0.1$; otherwise "NS" (= nonsignificant) is stated.

RESULTS

As already has been reported for sodium salicylate (3), several other salicylates are able to decrease the inorganic (radio)sulfate content in blood of mice after one single oral dose, see figure 1. Although demonstrating some delay in exerting their depressive effect - compared with that of sodium salicylate -

aspirin, diflunisal and benorylate, all in a dose of 1.25 mmol/kg, reduce serum sulfate very strongly to about 30% of the control level, 4 hours after administration. Another salicylate derivative, salicylamide, as well as paracetamol (acetaminophen) and phenol sodium, potent agents in depleting serum sulfate in rats and man by means of sulfate conjugation (14), not convincingly decrease the blood sulfate level, see figure 2. Albeit a small and significant reduction to 70% of control is achieved at 90 min after oral application of these drugs, it is not evident anymore at 4 hour post administration.

TABLE 1

Average standard deviation of control, blood sulfate values belonging to individual experiments (n=24)

Min after vehicle administration[a]	Mean control value[b]	Average standard deviation ± SD
30	100	16 ± 5
90	100	19 ± 7
240	100	23 ± 7

[a]Vehicle: 200 µl 0.1% methylcellulose.

[b]Expressed as ^{35}S radioactivity units per 10 µl blood.

Of interest, some other classes of antirheumatic drugs also reduce significantly the blood sulfate content, see figure 3. Phenylbutazone and its biotransformation product oxyphenbutazone, both exert a depressive effect after oral administration of a dose of 1 mmol/kg. However, oxyphenbutazone seems far less effective as phenylbutazone and provokes only at 90 min a significant depression of the blood sulfate content (35% reduction). Also chloroquine diphosphate, in an oral dose of 0.6 mmol/kg, slowly forces the sulfate level to go down to about 60% of control at 4 hour drug treatment.

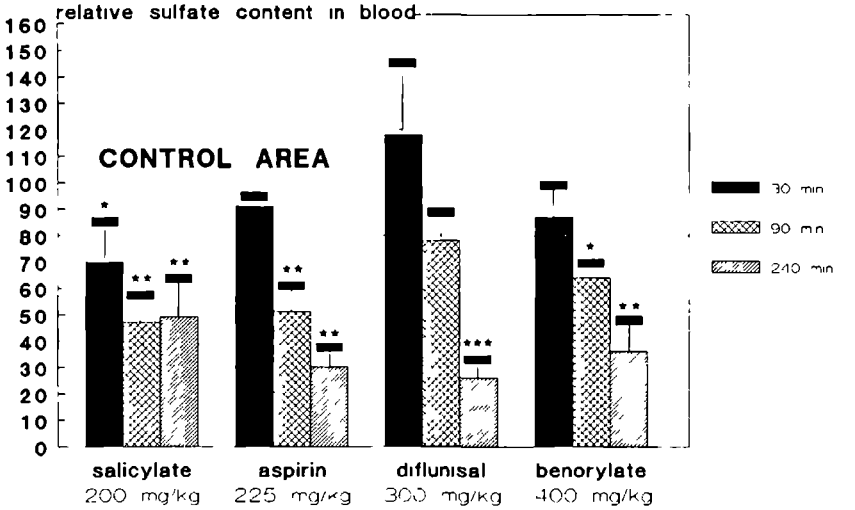

Figure 1.
Effect of salicylate analogues, all in a dose of 1.25 mmol/kg, on the inorganic (radio)sulfate content in blood at various times after oral administration. Results are depicted as vertical bars and represent the mean ± SD for five mice. For convenience, sulfate content in blood of control mice is stated as 100 ± 20 radioactivity units and is represented by the horizontal bar; see also statistical analysis section. $^{*}P \leq .05$; $^{**}P \leq .01$; $^{***}P \leq .001$

Quite notable is the depressive effect of tiaprofenic acid; a small dose of 0.06 mmol/kg is enough to decrease serum sulfate convincingly. Since such a small dose has such an impressive effect we further studied the sulfate lowering action of tiaprofenic acid by varying the amount of oral dose to be administered. Obviously, even one-third of the first-mentioned dose (0.02 mmol/kg) is able to decrease serum sulfate significantly at 4 hour post oral gavage, see table 2. Higher doses (0.12 and 0.35 mmol/kg) appear not to exceed the effects achieved with the 0.06 mmol dose. This finding is substantiated by measurements of endogenous, inorganic sulfate (13) in serum of 4 h samples (table 2). Some antirheumatic drugs - in the dosages we use - have no reducing effect on the inorganic sulfate content in mouse blood

and two of these, piroxicam and indomethacin, even tend to slow down significantly sulfate turnover as reflected in a retention of radiosulfate content in blood, see table 3.

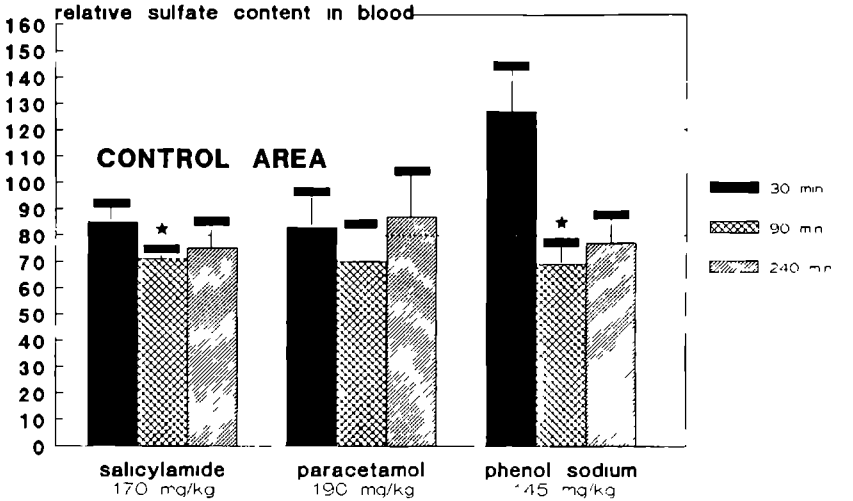

Figure 2.
Relative sulfate concentration in mouse blood, various times after administration of 1.25 mmol of commonly used sulfate lowering drugs/kg. See further legend to figure 1.

Pilot study with volunteers

In a small experiment a calcium salt of aspirin was examined for its eventual influence on serum sulfate in 5 healthy, adult, male volunteers, aged 27-44 year. One gram of the drug was taken by mouth at 0, 6, 11 and 17 hour. At 12 and 18 hour, blood was obtained and the amount of inorganic sulfate in the derived serum was quantified with our recently modified benzidine method (13). From serum obtained a day before - also at 12 and 18 hour - the normal concentration of sulfate in serum was determined. The results of this study are shown in table 4. There is no evidence for any difference in the serum sulfate content, indicating that aspirin does not affect inorganic sulfate in the circulation of normal, human subjects. The salicylate concentration in the serum

samples is of the same size as achieved therapeutically (15 - 40 mg%).

TABLE 2

Sulfate decreasing effect after oral gavage of tiaprofenic acid

Dose/kg (mg)	(mmol)	(Radio)sulfate content in 10 µl blood[a] 30 min	p^b	90 min	p^b	240 min	p^b	mM SO_4 at 240 min[c]
5	0.02	97±9	NS	97±18	NS	75±17	0.066	0.71±0.18
30	0.12	89±17	NS	78±14	0.018	49±10	0.001	0.58±0.10
90	0.35	101±26	NS	85±17	NS	55±13	0.003	0.53±0.11
control[b]		100±SD		100±SD		100±SD		0.92±0.16

[a]Values represent the mean ± SD for five mice and are expressed in radioactivity units.
[b]See statistical analysis section.
[c]Inorganic serum sulfate concentrations (mmol/l) are represented as the mean ± SD for 5 samples.

DISCUSSION

Except an abstract about this subject (15), this is the first document dealing with the existence of several serum sulfate lowering, antirheumatic drugs and their potential significance for articular cartilage integrity. From literature it is known that several drugs can be metabolized in vivo to sulfate conjugates, see table 5. Of these salicylamide and paracetamol are well known drugs which are often used even as a tool to decrease serum sulfate in rats (19) and dogs (20) for experimental studies. Moreover, these analgesic drugs also reduce serum sulfate in man (14,21). Recently, we have studied the effect of paracetamol on the serum sulfate content and its influence on articular cartilage in rats. Upon drug treatment the availability of inorganic sulfate in the circulation descended below a certain threshold. This phenomenon was shown to be responsible for the inhibition of

the production of newly formed proteoglycans in the cartilage (7). Daily administration of paracetamol to rats over a 4 week period, led to articular cartilage with a significant proteoglycan depletion (paper submitted for publication). From studies of the group of Maroudas (22) it is known that also proteoglycan synthesis in human articular cartilage is suppressed at too low concentrations of environmental sulfate; these circumstances might be created with paracetamol medication (2).

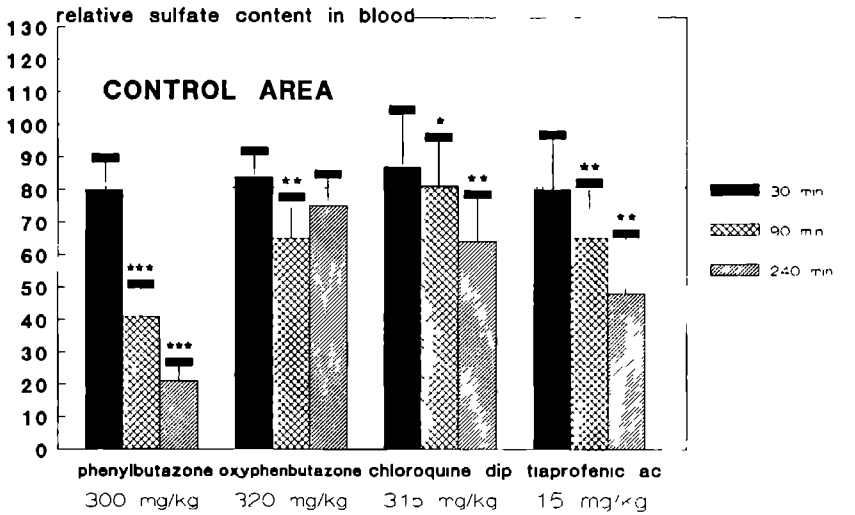

Figure 3.
Relative sulfate concentration in blood of mice various times after oral administration per kg body weight of: 0.1 mmol phenylbutazone or oxyphenbutazone, 0.6 mmol chloroquine diphosphate and 0.06 mmol tiaprofenic acid. See further legend to figure 1.

Another mechanism of decreasing inorganic sulfate in the circulation is via drug induced sulfate diuresis, first recognized with sodium salicylate in mice (3), of which examples will be discussed later.
Concerning our method to monitor the endogenous inorganic sulfate concentration in blood of mice, the following remarks: Thirty minutes after intraperitoneal administration of $^{35}SO_4$, the maximum concentration of ^{35}S in blood had already been passed (9). From

then on ^{35}S-kinetics follows the elimination kinetics of endogenous, inorganic sulfate, since more than 90% of ^{35}S is in the inorganic sulfate form (see Materials and Methods section).
Drug administration commences thirty minutes after $^{35}SO_4$ injection. Thus an eventual depressive effect on blood sulfate will be reflected in a decreased ^{35}S content compared to that of controls. However, if the drug itself is becoming sulfated or induces ^{35}S incorporation into organic blood components, ^{35}S-kinetics does not necessarily follow endogenous, inorganic sulfate elimination kinetics. Fortunately, this is not the case in our mouse model, since serum specimens derived from 4 hour's blood samples all contained more than 90% of ^{35}S associated with

TABLE 3

Effect of some antirheumatic drugs on the blood sulfate level in mice

Drug	Oral dose/kg		(Radio)sulfate content in 10 µl blood after drug treatment[a]					
	(mg)	(mmol)	30 min	p[b]	90 min	p[b]	240 min	p[b]
niflumic acid	305	1 08	101 ± 14	NS	85 ± 22	NS	116 ± 21	NS
diclofenac sodium	1 5	0 005	112 ± 22		115 ± 27		97 ± 20	
naproxen sodium	20	0.08	112 ± 25		126 ± 30		107 ± 22	
ketoprofen	50	0 20	100 ± 6		98 ± 15		88 ± 17	
ibuprofen	50	0 24	108 ± 19		113 ± 22		97 ± 19	
piroxicam	10	0 03	119 ± 15	0 037	145 ± 23	0.004	139 ± 31	0 060
indomethacin	2 0	0 006	124 ± 11	0.004	154 ± 24	0 002	146 ± 27	0 023
control[b]			100 ± SD		100 ± SD		100 ± SD	

[a]Values represent the mean ± SD for five mice and are expressed in radioactivity units.

[b]See statistical analysis section

inorganic sulfate and no significant difference in this matter existed between serum from drug-treated mice and that from controls (data not shown). The parallel between changes in ^{35}S content and endogenous sulfate is also illustrated in table 2.

TABLE 4

Sulfate concentration in human serum after aspirin intake

Sample	Sampling time	Serum concentration of[a]: Sulfate (mM)	Salicylate (mg%)[b]
Control	12 h	0.27 ± 0.05	-
	18 h	0.33 ± 0.07	-
Aspirin	12 h	0.31 ± 0.03	17 ± 5
	18 h	0.32 ± 0.04	20 ± 6

[a]Data represent the mean ± SD for five volunteers.
[b]Determined as described (3).

Not unexpected, the salicylate derivatives aspirin, diflunisal and benorylate, the latter is a conjugate of paracetamol and aspirin, provoke a decrease of inorganic sulfate in the circulation. We presume that the mechanism of action is similar to that of sodium salicylate, a drug which decreases serum sulfate in rats (4) and mice (3), by an unknown mode of action on the kidney, causing sulfate diuresis. The observed delay in action of the salicylate derivatives compared to that of sodium salicylate, could be explained by differences in absorption-rate kinetics or by the time lost for biotransforming (via hydrolysis) these agents to the active moiety, which possesses structures resembling the conjugated α-keto-enol system of salicylate. This same chemical structure can be adopted by phenylbutazone in a certain configuration (11,23), and we hypothesize that this property determines its sulfate lowering potential, also via a sulfaturic

effect. Yet, striking is the observation that the oxidized metabolite of phenylbutazone, oxyphenbutazone, does not possess a comparable sulfate lowering potency. The discrepancy between the mode of action of these two drugs cannot be explained by a higher contribution of ^{35}S in an organic blood component, e.g. sulfated oxyphenbutazon, since that would be less than 10% (see above).
Salicylamide, another salicylate derivative, just poorly decreased blood sulfate in mice as also did paracetamol and phenol sodium. Nevertheless, these agents have been used as a potent sulfate lowering device in many studies. Most phenolic substances, like the three in question here, are made hydrophylic primarily by conjugation with inorganic sulfate or glucuronic acid to eliminate them out of the body (14,24). An exception with respect to sulfate conjugation is salicylic acid, and probably some analogues, which despite of its phenolic nature cannot be sulfated in vivo (3). In species where sulfate conjugation is predominant over glucuronidation, the sulfation process is responsible for the decrease in circulating, inorganic sulfate observed after administration of salicylamide, paracetamol and phenol sodium. Of course, in this way only relatively high doses are able to decrease the sulfate level below a certain threshold, detrimental for cartilage. The doses we used were also used by other investigators (20,25,26). Apparently, an inefficient sulfate conjugation system in our mouse model could explain our findings; this would be in agreement with data from literature (25). In addition, our results indicate that the above mentioned drugs do not share an efficient sulfaturic effect.
Chloroquine diphosphate, from origin an antimalarial but today frequently used as a slow acting antirheumatic drug, also behaves like a sulfate lowering drug in our mouse model. Since here again most (i.e. $\geq$ 90%) of serum ^{35}S remains in the inorganic sulfate form, we assume that this drug is able to induce sulfate diuresis too.
Various drugs were devoid of any effect, see table 3. Piroxicam and indomethacin, both contrast sharply with the other drugs tested in just provoking a retention of radiosulfate in the blood of mice. We believe that these drugs also act on the kidney, but now in another way, i.e. retarding inorganic sulfate elimination. Of course, this would have no consequences for the proteoglycan

metabolism in cartilage cells.

TABLE 5

Some drugs serving as substrate for sulfate conjugation

Drug	References
Salicylamide	16, 14
Flufenamic acid	17
Paracetamol	14
Phenylbutazone	16, 17
Oxyphenbutazone	17
Prednisolone	16
Cortisone	16
Hydrocortisone	17
Chloroquine diphosphate	16, 17, 18
Hydroxychloroquine	16
Vitamin C, D	14

There is poor evidence in the literature about potential sulfate lowering drugs. Some drugs are reported to modulate the ^{35}S kinetics (tracer given as $^{35}SO_4$) in rat serum, as there are vitamin A, tolbutamide and triiodothyronine (4). Sulfaturic effects have been attributed to calcitonin (27), a small peptide with analgesic (28) and antiphlogistic properties (29); indeed high doses of sodium chloride are capable to exert sulfate

diuresis in dogs and rats (30,26). In addition, there is some evidence that steroids have a role in sulfate homeostasis, since castration or adrenalectomy of mice of either sex led to a significantly decreased, inorganic sulfate level, which was held responsible for the simultaneously observed suppression of proteoglycan synthesis in cartilage (31,32).

Although paracetamol and salicylamide medication leads to a decreased level of inorganic sulfate in serum of human subjects, this is apparently not the case with aspirin as indicated by our pilot study with volunteers; the same observations were made by other investigators (1). As already stated above, these salicylates cannot be conjugated with sulfate. Their sulfate lowering effects are ascribed to drug induced sulfate diuresis, at least in mice (3) and, as was very lately confirmed, in rats (5). To our knowledge, this latter phenomenon has never been described to occur in man. Perhaps it is not a side effect of antirheumatic drug therapy. Another possibility might still be that the dose of aspirin given was too low to stimulate the kidney to sulfate diuresis. In this regard the behaviour of tiaprofenic acid is interesting. At the very low dose of 0.02 mmol/kg, this drug significantly decreased the blood sulfate concentration by 25% ($p \leq 0.066$) in mice and this was potentiated at higher doses. The same drug load on the kidneys, experienced by mice, could presumable be obtained in arthritic patients on tiaprofenic acid medication. Anyway it remains to be seen how this subject develops in the future. We currently started with screening the sera of osteoarthritic and rheumatoid arthritic patients, which are on antirheumatic drug medication, for changes in the normal serum concentration of inorganic sulfate. In this respect it is important to note that women posses a slower turnover rate of inorganic sulfate than men (33); this consequently could lead to a delayed recovery of a temporary sulfate depletion. Yet, if ever a clinical active, sulfate lowering drug is found, its effect could always be counteracted by a fixed amount of inorganic sulfate or a precursor of this metabolite (e.g. cysteine), avoiding eventual drug mediated harm to cartilage. One potential candidate is already found, a nonprescription analgesic and antipyretic drug which is commonly used and freely obtainable over the whole world, paracetamol! Yet, it still remains to be seen if it

attacks cartilage, via sulfate lowering, in man.
Still an important lesson emerges from this study. Investigators examining direct effects of agents on proteoglycan turnover in experimental animals should be aware of two potential pitfalls:
1. If these agents depress the availability of inorganic sulfate they might indirectly suppress proteoglycan synthesis.
2. If radiosulfate is used as a tracer to quantify metabolic rates, changes in the specific radioactivity ($^{35}SO_4/SO_4$) in drug treated versus control animals may occur, depending upon the moment of ^{35}S-sulfate administration. As a consequence ^{35}S incorporation values must be corrected for these changes in specific activity, otherwise outcomes are misleading. These problems are of utmost relevance and should always be kept in mind when screening a new drug. The study of the in vivo effect of paracetamol on rat articular cartilage by Van der Kraan et al. (7) is instructive in this respect.

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CHAPTER 3

SYNTHESIS OF ABERRANT GLYCOSAMINOGLYCANS DURING CARTILAGE CULTURE IN "SULFATE FREE" MEDIUM

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Synthesis of aberrant glycosaminoglycans during cartilage culture in 'sulfate free' medium

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Summary

Incorporation of radiolabeled sulfate into glycosaminoglycans is a widely accepted assay to measure the rate of proteoglycan synthesis Although glycosaminoglycan synthesis is dependent on the quantity of inorganic sulfate available to proteoglycan synthesizing cells, 'sulfate free' medium is regularly used in studies regarding proteoglycan synthesis In this study murine patellar cartilage glycosaminoglycans synthesized under 'sulfate free' conditions were compared with those synthesized at physiological sulfate concentration Under 'sulfate free' conditions synthesis was not only decreased but low sulfated glycosaminoglycans were made that were not synthesized during incubation at physiological sulfate concentration The use of 'sulfate free' medium should be avoided in proteoglycan synthesis studies

Key words Sulfate, Glycosaminoglycan, Proteoglycan; Cartilage

Introduction

One of the major components of cartilage and other connective tissues are proteoglycans. Proteoglycans consist of one or more glycosaminoglycans covalently bound to a mainly linear protein core. Glycosaminoglycans are linear polymers of disaccharides. The glycosaminoglycan chains present in proteoglycans are sulfated. In connective tissue studies the incorporation of radiolabeled sulfate in glycosaminoglycans is a widely accepted quantitative measure of the rate of proteoglycan synthesis.

The synthesis of glycosaminoglycans in cartilage is dependent on the medium sulfate concentration in in vitro studies and on the serum sulfate concentrations in

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studies with laboratory animals [1–3]. Reduction of the medium sulfate concentration from 0.5 mM to 0 1 mM leads to a diminished incorporation of sulfate in human articular cartilage [1]. Medium sulfate concentrations below 0.5 mM result in a significantly decreased incorporation of sulfate in the glycosaminoglycans of anatomically intact patellar cartilage of the mouse [2]. Salicylate-induced sulfate depletion in mice leads to a diminished incorporation of sulfate in patellar glycosaminoglycans in vivo [2].

In studies of proteoglycan synthesis one should preferably use physiological medium sulfate concentrations. The various commercially available culture media differ markedly in their inorganic sulfate content as does the serum sulfate concentration in different species Human serum has the lowest sulfate concentration (0.3–0 4 mM) while rabbits have one of the highest serum sulfate concentrations (2 0 mM) [4,5] Cows (1 8 mM), fetal calfs (0 9 mM), dogs (1 2–1 8 mM) and mice (1 0 mM) have intermediate serum sulfate concentrations [2,5–8] However, little attention has been paid to the sulfate concentration in the medium in proteoglycan studies and 'sulfate free' medium is frequently used in these studies to increase the specific activity of [^{35}S]sulfate [9,10].

Experimental

We have studied the glycosaminoglycans synthesized in anatomically intact patellar cartilage of the mouse under 'sulfate free' conditions and physiological sulfate concentrations. The glycosaminoglycans were characterised by DEAE-anion-exchange chromatography and Sepharose CL 6B gel chromatography

The DEAE-Trisacryl chromatogram of glycosaminoglycans synthesized under physiological sulfate concentrations showed the presence of one pool of sulfated glycosaminoglycans (Fig 1) Treatment of glycosaminoglycans with chondroitinase ABC (1 0 U/ml in 0.01 M Tris-HCl, pH 8.0) resulted in the total disappearance of this glycosaminoglycan pool. We concluded that this pool represents chondroitin sulfate.

The DEAE-Trisacryl chromatogram of glycosaminoglycans synthesized under sulfate free conditions was very different from the chromatogram of glycosaminoglycans synthesized at physiological sulfate concentration (Fig 1) Primarily two glycosaminoglycan pools were synthesized under low sulfate conditions. A high and a low sulfated pool are produced predominantly by the patellar cartilage chondrocytes but glycosaminoglycans with intermediate levels of sulfation can also be seen in the chromatogram. All the glycosaminoglycans synthesized under low sulfate conditions were sensitive to treatment with chondroitinase ABC. The glycosaminoglycans present in the sulfate free chromatogram but not in the physiological sulfate chromatogram represent plausibly undersulfated chondroitine sulfate chains.

Results and Discussion

Based on the specific activity of [^{35}S]sulfate the incorporated quantity of [^{35}S]sulfate under sulfate free conditions is lower than expected (approximately

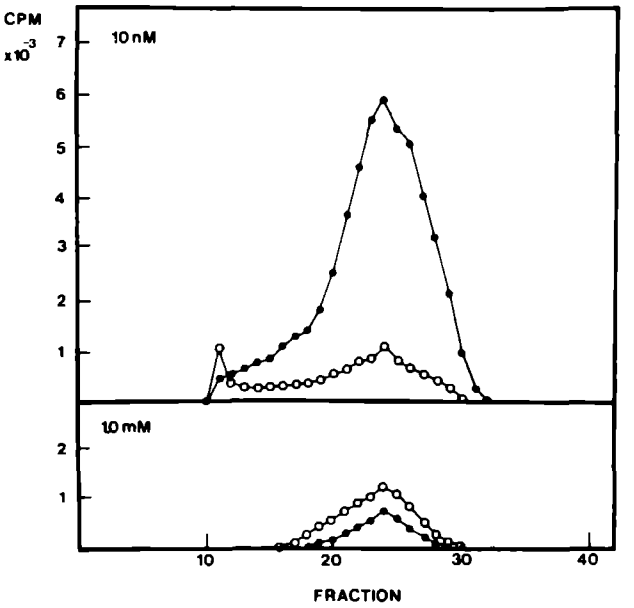

Fig. 1 DEAE-Trisacryl chromatogram of glycosaminoglycans synthesized in the presence of 1.0 mM (physiological) or 10 nM inorganic sulfate (sulfate free). Patellae from 7–9-week-old male C57Bl mice weighing 20–25 g were obtained according to the method of Van den Berg et al [11] Sulfate free BME-diploid medium (pH 7 4) was supplemented with L-glutamine (2 mM) and pyruvate (1 mM) and disodium sulfate was added to a final concentration of 1 0 mM or 10 nM. Groups of 10 patellae were incubated for 2 h in medium containing 100 μCi [^{35}S]sulfate (carrier free) and 200 μCi [^{3}H]glucosamine at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, patellae were washed and subsequently decalcified in 5% formic acid. The patellae were isolated from the surrounding tissue and digested with papain Treatment of the glycosaminoglycans with *Streptomyces* hyaluronidase was followed by lyophilization and redisolvation in elution buffer. A NaCl gradient (0.2–2.0 M in 1 mM HCl) was used to elute the glycosaminoglycans from the column. The specific activity of [^{35}S]sulfate was 100 Ci/mol in the experiments with 1.0 mM sulfate and 100×10^5 Ci/mol in the experiments with 10 nM ○, ^{3}H counts; ●, ^{35}S counts

7000-fold). This is due to the reduced glycosaminoglycan synthesis at low sulfate concentrations [1–3]. In our experiments (Figs. 1 and 2) the incorporation of [^{3}H]glycosamine was not diminished at low sulfate concentrations, but even slightly elevated at 10 nM sulfate compared to 1.0 mM sulfate. This can only for a small part be explained by the synthesis of low sulfated glycosaminoglycans at low sulfate concentrations. We think that this phenomenon can be explained by the following hypothesis. In the experiments with 1.0 mM sulfate the cartilage chondrocytes have a high rate of glycosaminoglycan synthesis and the glucose component of glucosamine will be derived mainly from the intracellular glycogen pool in the chondrocytes. A decreased synthesis of glycosaminoglycans, as a consequence of sulfate shortage, will result in a diminished need for saccharide precursors and therefore in a reduced degradation of glycogen to glucose. The reduced intracellular supply of glucose and consequently of unlabeled glucosamine will result in an increased

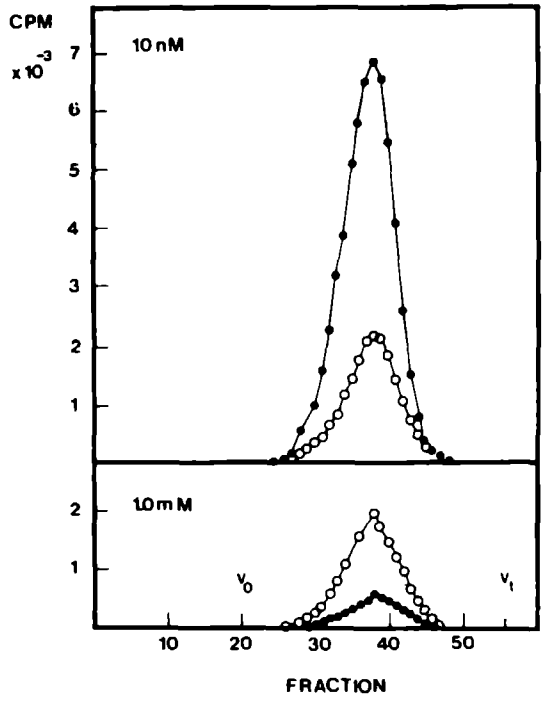

Fig. 2. Sepharose CL 6B chromatogram of glycosaminoglycans synthesized in the presence of 1.0 mM (physiological) or 10 nM inorganic sulfate (sulfate free) The incubation of the patellae and isolation of glycosaminoglycans is described in the legend of Fig. 1 An ammonium acetate buffer (pH 5 0) was used as elution buffer for the Sepharose CL 6B column Dextran blue (M_r 2×10^6) and [^{35}S]sulfate were used to determine the void volume and the total volume of the Sepharose CL 6B column. The specific activity of [^{35}S]sulfate was 100 Ci/mol in the experiments with 1 0 mM sulfate and 100×10^5 Ci/mol in the experiments with 10 nM sulfate. ○, ^{3}H counts, ●, ^{35}S counts.

specific activity of [^{3}H]glucosamine. This might explain the high incorporation of glucosamine in the experiments with 10 nM sulfate in the medium. The sulfate concentration had no effect on the length of the chondroitine sulfate chains (Fig. 2). The glycosaminoglycans synthesized under both sulfate free or the physiological sulfate concentration had a K_{av} of 0.5.

The results of this study demonstrate the importance of the sulfate concentration in the medium in proteoglycan synthesis studies. The use of sulfate free medium leads to decreased synthesis and to production of glycosaminoglycans not made in the presence of physiological sulfate concentrations during an incubation period of only 2 h. The aberrant glycosaminoglycan synthesis under low sulfate conditions might not only lead to inaccurate conclusions about proteoglycan synthesis itself but also to inaccurate conclusions about the action of proteoglycan synthesis modifying agents. At present we study the effect of less extreme sulfate concentration (0.1–1.0 mM) on glycosaminoglycan metabolism. Incubation of murine patellae in medium with less than 0.5 mM sulfate led to an increased susceptibility of the glycosaminoglycan synthesis to salicylate [3].

Remarks and a recommendation on a currently used method

Synthesis of glycosaminoglycans can be studied by quantitation of the incorporation of radiolabeled sulfate or glucosamine in these glycosaminoglycans. Culture media with very low inorganic surfate concentrations (sulfate free medium) are frequently used in these studies to increase the specific activity of [^{35}S]sulfate. In this study we show that the use of sulfate free medium leads to the synthesis of glycosaminoglycans not synthesized under physiological conditions. So, we recommend that physiological sulfate concentrations in culture media should be used in glycosaminoglycan synthesis studies.

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CHAPTER 4

THE EFFECT OF LOW SULFATE CONCENTRATIONS ON THE GLYCOSAMINOGLYCAN SYNTHESIS IN ANATOMICALLY INTACT ARTICULAR CARTILAGE OF THE MOUSE

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The Journal of Orthopaedic Research: in press

The Effect of Low Sulfate Concentrations on the Glycosaminoglycan Synthesis in Anatomically Intact Articular Cartilage of the Mouse

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Summary. We have studied the effect of environmental sulfate concentration on the glycosaminoglycan synthesis of anatomically intact patellar cartilage of the mouse in vitro. Incubation of mouse patellae in medium with sulfate concentrations below 0.5 m*M* resulted in a diminished incorporation of sulfate but in unaltered incorporation of glucosamine. This suggested the synthesis of undersulfated glycosaminoglycans under these conditions. We characterized glycosaminoglycans synthesized at three different sulfate concentrations: a sulfate concentration physiological for the mouse (1 0 m*M*), a sulfate concentration in the range where sulfate incorporation was strongly diminished (0.1 m*M*), and an extremely low sulfate concentration (10 n*M*) Analysis of glycosaminoglycan disaccharides and DEAE anion chromatography of the glycosaminoglycans could not confirm the synthesis of undersulfated glycosaminoglycans at 0 1 m*M*. The chromatogram of glycosaminoglycans synthesized in medium containing 10 n*M* showed the presence of a very low sulfated glycosaminoglycan pool not observed at higher medium sulfate concentrations. Intermediately sulfated glycosaminoglycans were also synthesized during incubation with 10 n*M* sulfate. So, our data indicate that only very low sulfate concentrations in the medium lead to the synthesis of undersulfated glycosaminoglycans and that the sulfation mechanism of murine patellar cartilage chondrocytes does not seem to fit completely in an "all-or-nothing" pattern. **Key Words:** Sulfate—Cartilage—Glycosaminoglycans—Mouse—Articular

Sulfate groups on the glycosaminoglycan chains of proteoglycans are essential for the pressure-resistant function of cartilage. Undersulfated glycosaminoglycans are synthesized by the cartilage of brachymorphic mice resulting in a disproportional short stature characterized by shortened long bones (18). This mutant is defective in the synthesis of sufficient amounts of 3'-phosphoadenosine 5'-phosphosulfate (21,25). Cartilage is the tissue affected primarily, owing to the high rate of glycosaminoglycan synthesis (19). Patients with Lowe's syndrome (oculocerebrorenal syndrome), characterized by cataracts, renal abnormalities, growth failure, rickets, arthropathy, and mental retardation, excrete abnormal quantities of undersulfated glycosaminoglycans in their urine (1,14). Fibroblasts from these patients synthesize undersulfated chondroitin and dermatan sulfate (10). Both humans and dogs with spondyloepiphyseal dysplasia are known to synthesize undersulfated glycosaminoglycans (3,16,17).

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One possible cause of the synthesis of undersulfated glycosaminoglycans could be a limited supply of inorganic sulfate to the cells Incubation of embryonic chick cartilage at low sulfate concentrations leads to the synthesis of both fully and nonsulfated chondroitin sulfate chains (13,24) This "all-or nothing" sulfation pattern was described earlier for the synthesis of chondroitin sulfate in cell-free systems (4) The sulfation of chondroitin in mesenchyme cultures of brachymorphic mice also seems to follow an all-or-nothing mechanism (19) Recently it was found that sulfation of chondroitin by bovine aortic endothelial cells at intermediate sulfate concentrations did not fit in an all-or-nothing mechanism (12) Engelbreth-Holom-Swan tumor cells synthesize two different pools of heparan sulfate chains a low sulfated and a high sulfated pool The high sulfated pool is synthesized predominantly under high-medium sulfate conditions, while the low sulfated pool is synthesized primarily under low sulfate conditions (26) The proportion of dermatan sulfate to chondroitin sulfate synthesized by human fibroblasts can also be affected by the medium sulfate concentration A low medium sulfate concentration favors the formation of chondroitin sulfate (23)

In earlier studies we found that salicylate-induced sulfate depletion in mice leads to a diminished incorporation of sulfate in patellar glycosaminoglycans (5,6) We examined the synthesis of glycosaminoglycans by anatomically intact articular cartilage of the mouse to answer the question of whether only synthesis is decreased or whether undersulfated glycosaminoglycans are also synthesized at decreased sulfate concentrations When calculated by the incorporation rate of glucosamine and sulfate into newly synthesized glycosaminoglycans, reduction of the medium sulfate concentration from 1 0 to 0 1 m*M* resulted in a 50% decrease in sulfate incorporation but no decrease in glucosamine incorporation However, chromatographic analysis of the newly synthesized glycosaminoglycans did not confirm the presence of undersulfated or nonsulfated glycosaminoglycan chains Undersulfation of glycosaminoglycans occurred only at very low sulfate concentrations in the medium The discrepancy between radiolabel incorporation and chromatography might be caused by alterations in the specific activity of the labels used under conditions of a diminished synthesis of glycosaminoglycans

MATERIALS AND METHODS

Materials

[^{35}S]Sulfate (carrier-free, 1,200 Ci/mmol spec act) and D-[6 ^{3}H]glucosamine (40 Ci/mmol spec act) were obtained from the Radiochemical Centre (Amersham) BME-diploid medium (sulfate-free) and glutamine were purchased from Flow Laboratories and pyruvate from Boehringer Mannheim Chondroitinase ABC from *Proteus vulgaris*, hyaluronidase from *Streptomyces hyalurolyticus*, and papain (type IV, double crystallized) were obtained from Sigma Sepharose CL 6B was obtained from Pharmacia and DEAE Trisacryl from LKB Chondroitin was purchased from Serva and 24-well cluster dishes from Costar Solulyte was purchased from J T Baker Chemicals

Dissection of Patellae

Patellae were obtained from 7- to 9-week-old male C57Bl10 mice in good health and weighing 20–25 g Mice were killed by cervical dislocation and the whole patellae, with a standard amount of surrounding tissue, were dissected from the knee joints according to the method of van den Berg et al (27) The incorporation of [^{35}S]sulfate in the patellae and surrounding tissue is shown in Fig 1 Radiolabel was found almost exclusively in the cartilage of the patellae Hardly any label was found in the underlying bone and bone marrow cells (<1%) (7) Note the absence of epiphyseal cartilage in the patellae After incubation of the patellae with surrounding tissue in culture medium, they were carefully isolated from the surrounding tissue and only the glycosaminoglycan synthesis in the patellae was examined

Incubation of Patellae

BME-diploid medium (pH 7 4) was supplemented with L-glutamine (2 m*M*) and pyruvate (1 m*M*) Groups of six patellae with surrounding tissue were incubated in BME diploid medium (200 μl/patella) with sulfate concentrations ranging from 0 1 to 1 2 m*M* for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 After 2 h 20 μCi/ml [^{35}S]sulfate and 40 μCi/ml [^{3}H]glucosamine were added to the incubation medium Glycosaminoglycan synthesis is linear within 5 min in our incubation system (data not shown), so 2 h preincubation is sufficient to equilibrate the sulfate concentrations across the tissue and cell membrane barrier

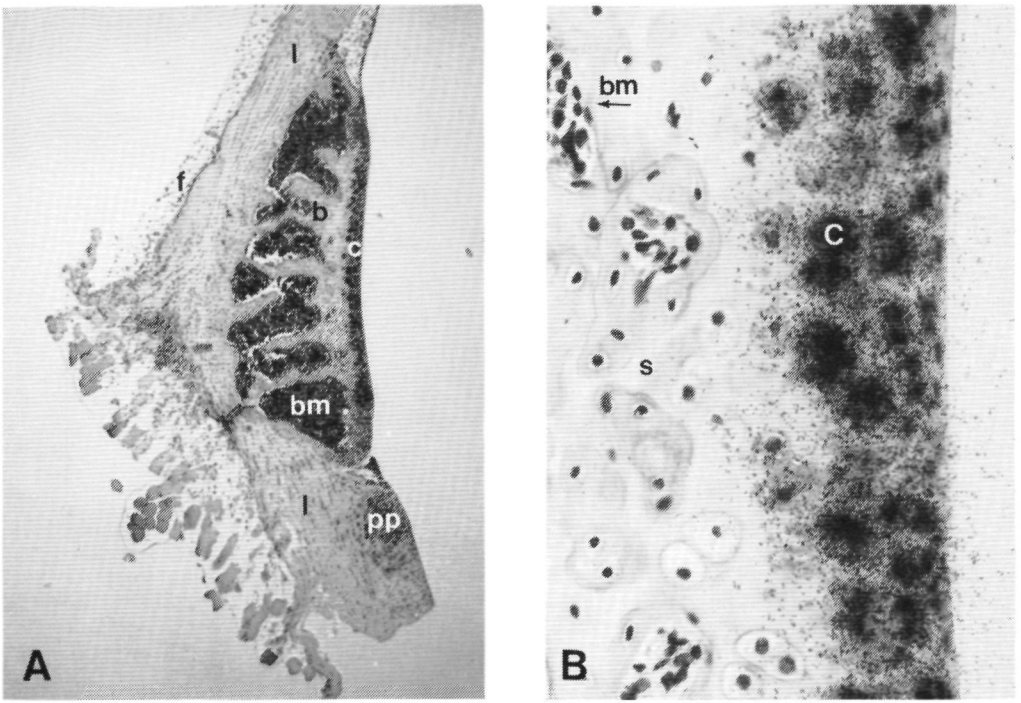

FIG. 1. Autoradiograph of a mouse patella surrounded by connective tissue labeled with [^{35}]sulfate in vitro. Note the absence of epiphyseal cartilage. c, cartilage; b, bone; bm, bone marrow; pp, patellar plate; l, ligament; f, fat tissue; s, subchondral bone. Hematoxylin and eosin; ×40 **(A)**, ×400 **(B)**.

We investigated the effect of sulfate concentration in the medium on the recycling of sulfate derived from degraded glycosaminoglycans. Patellae were incubated for 2 h in medium containing 1.0 m*M* sulfate and 20 μCi/ml [^{35}S]sulfate. This was followed by a 16-h incubation period in medium with various sulfate concentrations without label.

Isolation of Glycosaminoglycans

After incubation the patellae were washed twice with physiological saline to remove most nonincorporated label and fixed in 4% phosphate-buffered formaldehyde (pH 7.0). The whole patellae were isolated from the surrounding tissue after overnight decalcification in 5% formic acid. A papain mixture consisting of 1 mg/ml papain, 0.2 *M* NaCl, 0.1 *M* Na-acetate, 10 m*M* L-cysteine hydrochloride, 50 m*M* Na_2-EDTA, and 50 μg/ml chondroitin sulfate carrier (pH 6.0) was used to digest formaldehyde-fixed patellae (200 μl/patellae) at 60°C overnight.

Undigested patellar remnants were spun down. To 150 μl supernatant of patella digest, 150 μl 0.2% cetylpyridinium chloride was added. After 2 h at 37°C, the precipitate was centrifuged at 10,000 *g* for 15 min at 37°C. The supernatant was discarded and the precipitate was washed twice with 0.5 ml 0.05% cetylpyridinium chloride. The pellet was solubilized for 2 h in 0.5 ml Solulyte at 60°C and after addition of scintillation fluid analyzed by a liquid scintillation counter. Appropriate corrections were made for the ^{3}H and ^{35}S channel overlap.

Analysis of Glycosaminoglycans by Column Chromatography

Groups of 10 patellae were pooled and incubated for 2 h in BME-diploid medium containing 10 n*M*,

0 1 m*M*, or 1 0 m*M* sulfate supplemented with 100 μCi/ml [^{35}S]sulfate and 200 μCi/ml [^{3}H]glucosamine The patellae were preincubated for 2 h in medium without label. After papain digestion as described above and spinning down of the undigested remnants, the digest supernatants were dialyzed for 72 h against tap water This was followed by lyophilization and redissolution of the glycosaminoglycans in the elution buffers used during chromatography.

The glycosaminoglycans of 10 pooled patellae were applied to a DEAE-Trisacryl anion exchange column (1 × 15 cm) that had been equilibrated with 200 ml 0 2 *M* NaCl in 1 m*M* HCl. A linear gradient of 0.2–2 0 *M* NaCl in 1 m*M* HCl was used to elute the glycosaminoglycans, as described by Ito et al. (13). After each run the column was washed with 100 ml 0 2 *M* NaCl in 1 m*M* HCl. The flow was 20 ml/h and fractions of 2 ml were collected. The fractions were assayed for radiolabeled glycosaminoglycans by liquid scintillation counting

The glycosaminoglycans of 10 pooled patellae were also applied to a Sepharose CL 6B column (100 × 0 7 cm) A 0.1 *M* ammonium acetate buffer (pH 5 0) was used as eluant. The flow was 15 ml/h and fractions of 1.25 ml were collected and assayed for radioactively labeled glycosaminoglycans. The void volume (V_0) of the column was determined by dextran blue (M_r 2 × 10^6) and the total volume (V_t) by $Na_2[^{35}S]SO_4$.

Enzymatic Digestion of Glycosaminoglycans

The hyaluronate fraction of the glycosaminoglycans was digested by the specific *S. hyaluronidase* to demask the synthesized chondroitin. Hyaluronate elutes in the same fractions as chondroitin. Glycosaminoglycans were dissolved in 1 ml 0 02 *M* Na-acetate buffer (pH 5) containing 10 U/ml enzyme and incubated for 24 h at 37°C After incubation the samples were dialyzed for 48 h against tap water and subsequently lyophilized.

To characterize the synthesized glycosaminoglycans, chondroitin sulfate was degraded after redissolution of the glycosaminoglycan samples in 1 ml 0 01 *M* Tris-HCl (pH 8) with 1 0 U/ml chondroitinase ABC for 24 h at 25°C. This was followed by dialysis for 48 h against tap water and lyophilization of the samples.

Analysis of Glycosaminoglycan Disaccharides with High Performance Liquid Chromatography

Patellae were incubated with [^{35}S]sulfate and [^{3}H]glucosamine as described above. Individual patellae were incubated with papain and subsequently treated with *S. hyaluronidase*. After dialysis and lyophilization, the glycosaminoglycans were treated with chondroitinase ABC. The proportions of radiolabeled nonsulfated disaccharides, 4-sulfated disaccharides, and 6-sulfated disaccharides were determined by the method described by Hjerpe et al. (11) A Radialpak NH_2 high performance liquid chromatography (HPLC) column was used to separate the disaccharides.

Statistical Analysis

Statistical analysis of data was performed by the double-sided Student *t* test.

RESULTS

The incorporation of sulfate and glucosamine in the glycosaminoglycans of patellar cartilage incubated in medium with various sulfate concentrations is shown in Fig 2 (isolation of cartilage from the patellae gave similar results as whole patellae, data not shown) The incorporated quantity was calculated from the incorporation of labeled sulfate and glucosamine corrected for the specific activities of these precursors used in the various experimental conditions. The incorporation of sulfate significantly decreased when the medium sulfate concentration was reduced from 1 2 to ≤0.4 m*M* ($p < 0.001$). The incorporation of glucosamine in the newly synthesized glycosaminoglycans was not diminished by reduction of the medium sulfate concentration and even seemed to increase at very low

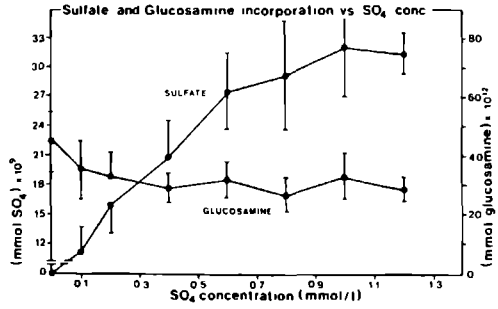

FIG. 2. Sulfate and glucosamine incorporation into patellar glycosaminoglycans at various sulfate concentrations Results are expressed as millimoles of precursors incorporated in 2 h per patella and represent the means ± SD of at least five patellae

sulfate concentrations These results point to the synthesis of undersulfated glycosaminoglycans in vitro at lower than physiological sulfate concentrations The physiological sulfate concentration of the mouse is 1 0 m*M* (8)

To study the effect of the sulfate concentration in the medium on the recycling of sulfate derived from degraded glycosaminoglycans, mouse patellae were incubated for 2 h in medium with 1.0 m*M* sulfate and [^{35}S]sulfate This was followed by a 16-h chase in medium with various sulfate concentrations without label The results are shown in Table 1 The medium sulfate concentration had no effect on the degradation of newly synthesized glycosaminoglycans, moreover, the results do not indicate an elevated recycling of sulfate derived from degraded glycosaminoglycans at low sulfate concentrations

The results described above seem to imply the synthesis of undersulfated glycosaminoglycans or of a nonsulfated glycosaminoglycan pool besides the normal sulfated pool under low sulfate conditions To investigate the nature of the glycosaminoglycans synthesized under these sulfate concentrations, we have analyzed glycosaminoglycans synthesized in the presence of 1 0 m*M*, 0 1 m*M*, and 10 n*M* sulfate by DEAE-Trisacryl anion exchange chromatography. Both the ^{3}H counts and the ^{35}S counts were recovered for 85–95% after chromatography.

The chromatograms of the glycosaminoglycans showed two peaks (Fig. 3): a very low or nonsulfated peak that eluted in fraction 10 (0 38 *M* NaCl) and a highly sulfated peak that eluted in fraction 24–25 (0 66 *M* NaCl) After treatment of the samples with chondroitinase ABC, both peaks disappeared from the chromatograms We found no indication of undersulfation in the chromatogram of the glycosaminoglycans synthesized in the presence

TABLE 1 *Effect of sulfate concentration in the medium on the degradation of newly synthesized glycosaminoglycans of mouse patellae*

Sulfate concentration (m*M*)	Chase period (h)	^{35}S content ± SD (cpm/patella)
1 0	0	330 ± 60
1 0	16	161 ± 34
0 4	16	150 ± 19
0 1	16	186 ± 24
0 0	16	171 ± 26

Mouse patellae were incubated for 2 h in medium with 1 0 m*M* sulfate and 20 μCi/ml [^{35}S]sulfate and chased afterward in medium with various sulfate concentrations without label Groups of six patellae were used

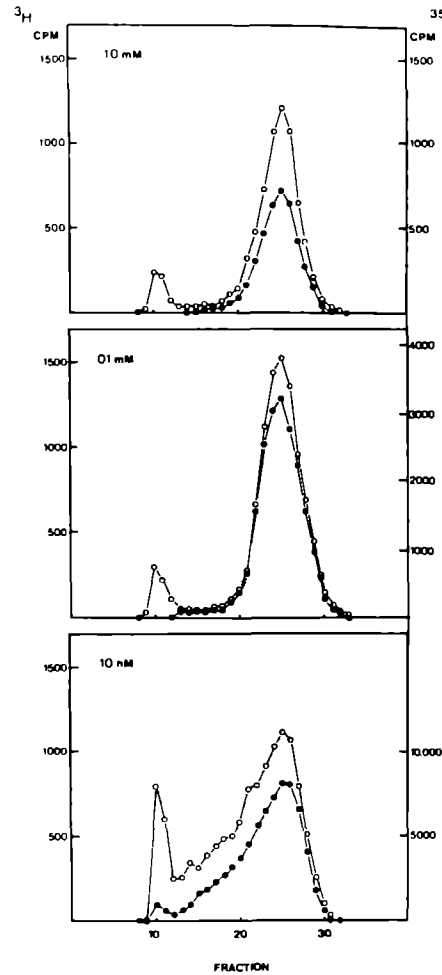

FIG. 3. DEAE-Trisacryl chromatogram of patellar glycosaminoglycans synthesized at 1 0 m*M*, 0 1 m*M*, and 10 n*M* sulfate Glycosaminoglycans of 10 murine patellae were pooled The specific activity of [^{35}S]sulfate was 100, 1 000, and 100 × 10^{5} Ci/mol respectively, in the experiments with 1 0 m*M*, 0 1 m*M*, and 10 n*M* sulfate in the incubation medium (○), ^{3}H, (●), ^{35}S

of 0 1 m*M* sulfate. The chromatograms of 1.0 and 0.1 m*M* were similar. Neither a shift of the sulfated glycosaminoglycan peak nor an increase in the proportion of the nonsulfated peak was observed. However, calculation of the quantity of incorporated sulfate showed a 60% decrease at 0.1 m*M* sulfate as observed earlier (Fig. 2) Incubation of patellae in medium containing 10 n*M* sulfate re-

sulted in the synthesis of deviant glycosaminoglycans The proportion of the very low sulfated glycosaminoglycan peak increased and the highly sulfated peak showed a widening on the left side of the chromatogram, indicating the presence of less sulfated glycosaminoglycan chains

Treatment of the glycosaminoglycan samples with hyaluronidase from *Streptomyces* resulted in the chromatograms shown in Fig 4 The glycosaminoglycan peak eluting at 0 38 *M* NaCl disappeared completely in the chromatogram of 1 0 m*M* and almost completely in the chromatogram of 0 1 m*M* This demonstrates that the first peak in the chromatogram of 1 0 and 0 1 m*M* is hyaluronate The first peak of 0 1 *mM* also contained a small fraction of chondroitin The first peak in the chromatogram of 10 n*M* was affected to only a small extent after the treatment with hyaluronidase This peak represents, besides hyaluronate, a very low or nonsulfated glycosaminoglycan pool, in all probability chondroitin

These results were confirmed by analysis of the glycosaminoglycan disaccharides by HPLC (Table 2) The glycosaminoglycan pool synthesized in the presence of 10 n*M* showed a significantly higher proportion of nonsulfated disaccharides than the glycosaminoglycan pools synthesized in the presence of 0 1 or 1 0 *mM* sulfate ($p < 0\ 001$) In addition, significant reductions in pool size of chondroitin-6-sulfate ($p < 0\ 05$) and chondroitin-4-sulfate ($p < 0\ 001$) were noticed only at 10 n*M* sulfate There was no significant difference in the proportion of nonsulfated disaccharides comparing 0 1 and 1 0 m*M* sulfate

The length of the chondroitin sulfate chains was not changed by a decrease of the sulfate concentration from 1 0 m*M* to 10 n*M* (Fig 5) Hyaluronate (first peak) eluted in the void volume of the Sepharose CL 6B column, while the chondroitin sulfate chains had a K_{AV} of 0 50

DISCUSSION

Incubation of mouse patellae in medium containing <0 5 m*M* sulfate led to a decreased incorporation of sulfate but had no effect on the incorporation of glucosamine However, the synthesis of undersulfated glycosaminoglycans could be confirmed only at very low sulfate concentrations in the medium (10 n*M*) Incubation of patellae in medium containing 0 1 m*M* sulfate did result only in the synthesis of a minor extent of undersulfated glycosaminoglycans Earlier studies revealed that oral administration of sodium salicylate to mice leads to serum sulfate depletion (5,6) This depletion (60%) will diminish the rate of glycosaminoglycan synthesis but will not alter the quality of the glycosaminoglycans synthesized

We examined the effect of decreased medium sul-

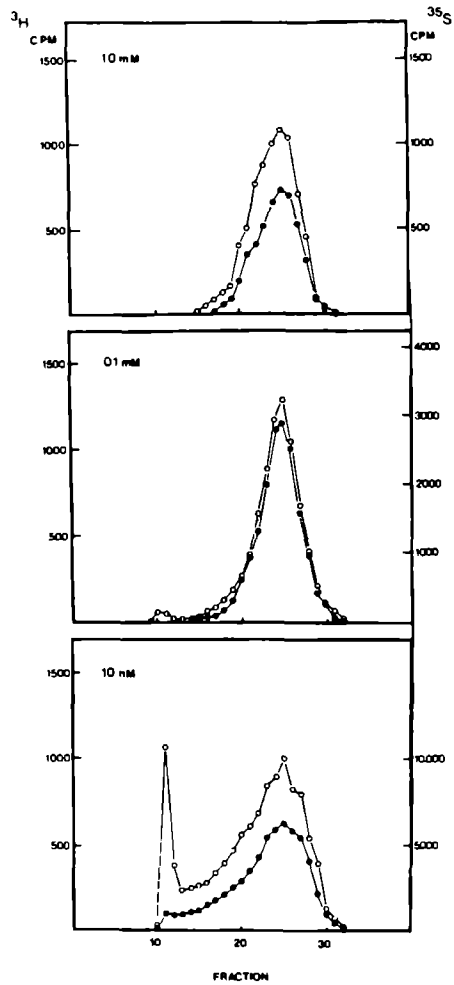

FIG 4 DEAE Trisacryl chromatogram of patellar glycosaminoglycans synthesized at 1 0 m*M* 0 1 m*M* and 10 n*M* sulfate after treatment with *S hyaluronidase* Glycosaminoglycans of 10 murine patellae were pooled The specific activity of [^{35}S]sulfate in the experiments is indicated in the legend of Fig 3 (○) ^3H (●) ^{35}S

TABLE 2. *Effect of sulfate concentration in the medium on the disaccharide proportion of newly synthesized patellar glycosaminoglycans*

Sulfate concentration	Nonsulfated disaccharide (%)	4-Sulfated disaccharide (%)	6-Sulfated disaccharide (%)
10 n*M*	26 6 ± 2 7	57 2 ± 2 1	16 2 ± 1 7
0 1 m*M*	16 6 ± 1 7	66 0 ± 1 3	18 4 ± 1 5
1 0 m*M*	15 0 ± 0 7	65 4 ± 1 3	19 6 ± 1 8

Glycosaminoglycans were treated with hyaluronidase and dialyzed After lyophilization and redisolution, the glycosaminoglycans were treated with chondroitinase ABC and analyzed in the high performance liquid chromatography system The results are expressed as means ± SD of five patellae

fate concentrations on the synthesis of glycosaminoglycans in anatomically intact articular cartilage. We used the whole patella of the mouse because of the integrity of the cartilage extracellular matrix and the chondrocytes. The environment of the chondrocytes is not changed compared with the in vivo situation (7). Slicing of articular cartilage, causing disruption of the collagen meshwork, or isolation of chondrocytes might alter the chondrocyte metabolism.

The incorporation rate of sulfate and glucosamine into glycosaminoglycans seemed to indicate the synthesis of undersulfated glycosaminoglycans at sulfate concentrations below 0.5 m*M*. However, the DEAE-Trisacryl chromatogram of glycosaminoglycans synthesized in the presence of 0.1 m*M* sulfate showed the presence of undersulfated glycosaminoglycans only to a minor extent, not enough to explain the diminished sulfate incorporation. Incubation of patellae in medium with 10 n*M* sulfate did result in the synthesis of undersulfated glycosaminoglycans. Sobue et al. (24) observed undersulfation of glycosaminoglycans synthesized by embryonic chick cartilage at medium sulfate concentrations below 30 μ*M*. On the other hand, sulfate concentrations of >0.11 m*M* were required for the synthesis of normal sulfated glycosaminoglycans of bovine aortic endothelial cells (14).

Decrease of the sulfate concentration below 0.5 m*M* led to a decreased incorporation of sulfate in glycosaminoglycans. Similar results are found by Maroudas and Evans (15) for the incorporation of sulfate in slices of human and bovine articular cartilage glycosaminoglycans and by Bayliss et al. (2) for rabbit annulus cartilage. Experiments with anatomically intact patellar cartilage of Wistar rats also resulted in a diminished incorporation of sulfate in medium containing <0.5 m*M* sulfate (our observation, results not shown). A decreased sulfate incorporation is considered to be a reflection of a diminished glycosaminoglycan synthesis. An apparent decrease of sulfate incorporation might also be caused by an elevated recycling of sulfate derived from degraded unlabeled glycosaminoglycans at decreased sulfate concentrations. The medium sulfate concentration had no effect on the release of ^{35}S counts from labeled glycosaminoglycans, as would

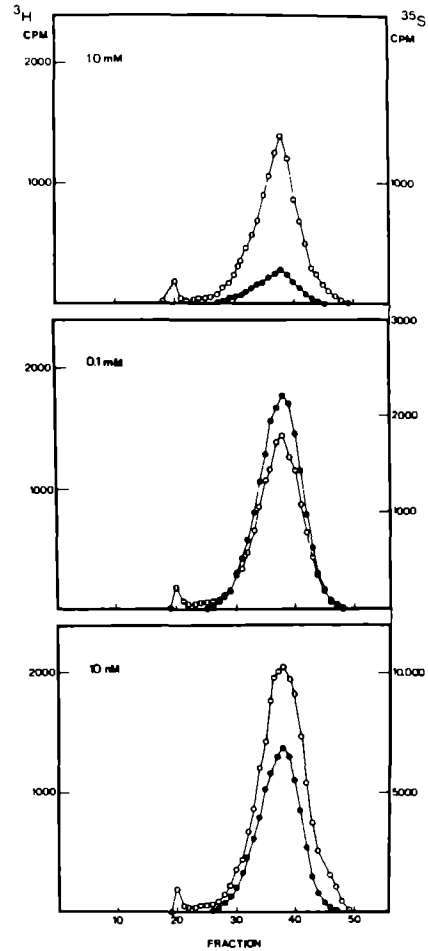

FIG. 5. Sepharose CL 6B chromatogram of patellar glycosaminoglycans synthesized at 1 0 m*M*, 0 1 m*M*, and 10 n*M* sulfate Glycosaminoglycans of 10 murine patellae were pooled The specific activity of [^{35}S]sulfate in the experiments is indicated in the legend of Fig 3 (○), ^{3}H, (●), ^{35}S

be expected when sulfate recycling changes as a function of the medium sulfate concentration (Table 1) Also, recycling of sulfate derived from lysosomally degraded glycosaminoglycans was not observed using human fibroblasts (20).

Inorganic sulfate is not the only sulfate source that can be used for the sulfation of glycosaminoglycans. An accelerated turnover of sulfur-containing amino acids at low environmental sulfate concentrations could also lead to an apparent reduction of sulfate incorporation Sulfate transport–deficient mutants of Chinese hamster ovary cells obtain their sulfate for the sulfation of glycosaminoglycans from the catabolism of cysteine (9). However, cartilage-synthesizing cells make at least 1,000-fold more glycosaminoglycans than Chinese hamster ovary cells (9). Consequently, the sulfate supply from degradation of cysteine or methionine will not be sufficient to affect the sulfation of glycosaminoglycans in chondrocytes considerably

Other authors also observed a decreased sulfate incorporation and an unaltered glucosamine incorporation in their experiments. Treatment of cartilage epiphyses from chick embryos with 6-aminonicotinamide resulted in 30% decrease of sulfate incorporation but had no effect on glucosamine incorporation (22). A decreased sulfate incorporation at sulfate concentrations below 0 5 m*M* plausibly reflects a diminished glycosaminoglycan synthesis The unaltered incorporation of glucosamine could be the result of changes in the intracellular specific activity of [^{3}H]glucosamine Intracellular glucosamine might be derived from glycogen present in the chondrocytes when the demand of glucosamine is high as a result of a high rate of glycosaminoglycan synthesis. A decreased synthesis of glycosaminoglycans as a consequence of decreased medium sulfate concentrations or glycosaminoglycan synthesis–inhibiting compounds, like 6-aminonicotinamide, would result in a reduced breakdown of glycogen to glucose. The reduced intracellular supply of glucose and by this of unlabeled glucosamine will result in an increased specific activity of [^{3}H]glucosamine. This could explain the apparent undisturbed incorporation of glucosamine although the synthesis of glycosaminoglycans is inhibited.

Other investigators have found, in culture systems differing from the one we have used in our experiments, an all-or-nothing pattern of glycosaminoglycan sulfation (4,13,19,24). The sulfation of chondroitin sulfate chains in embryonic chick epiphyseal cartilage and embryonic chicken sternum cartilage occurred in such a fashion (13,24), similar results were observed when a microsomal preparation of chick embryo epiphyseal cartilage was used (4). Limb mesenchyme cultures from brachymorphic mice showed also an all-or-nothing mechanism of sulfation, as reported by Penneyacker et al. (19). The sulfation of glycosaminoglycans by chondrocytes of murine articular cartilage, as described above, did not seem to follow this sort of pattern The sulfation mechanism had a predisposition for the synthesis of a highly sulfated chondroitin sulfate pool and a very low sulfated chondroitin pool. However, also intermediately sulfated glycosaminoglycans chains could be observed in the chromatogram of glycosaminoglycans synthesized in the presence of 10 n*M* sulfate

The results of this study show that the medium sulfate concentration is an important determinant of glycosaminoglycan synthesis. Many investigators use "sulfate-free" medium to increase the specific activity of [^{35}S]sulfate. This will undoubtedly lead to the synthesis of glycosaminoglycans not synthesized under physiological sulfate concentrations and will result in inaccurate conclusions about the synthesis of glycosaminoglycans.

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CHAPTER 5

INHIBTION OF GLYCOSAMINOGLYCAN SYNTHESIS IN ANMATOMICALLY INTACT RAT PATELLAR CARTILAGE BY PARACETAMOL-INDUCED SERUM SULFATE DEPLETION

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INHIBITION OF GLYCOSAMINOGLYCAN SYNTHESIS IN ANATOMICALLY INTACT RAT PATELLAR CARTILAGE BY PARACETAMOL-INDUCED SERUM SULFATE DEPLETION

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Abstract—We have studied the effect of low sulfate concentrations on the glycosaminoglycan synthesis in rat patellar cartilage *in vivo* as well as *in vitro* The oral administration of 200 mg/kg paracetamol to male Wistar rats resulted in a significant reduction of the serum sulfate concentration Reduced serum sulfate availability resulted in a 34% decrease of glycosaminoglycan synthesis in patellar cartilage This is due to sulfate depletion since paracetamol had no direct effects on glycosaminoglycan synthesis and a slight but significant inhibitory effect on the catabolism of radiolabeled glycosaminoglycans *in vitro*

The glycosaminoglycans synthesized at low sulfate concentrations *in vivo* were similar to the glycosaminoglycans synthesized at physiological sulfate concentrations Studying the effect of sulfate availability *in vitro* on glycosaminoglycan synthesis in patellar cartilage we found that incubation of rat patellae in medium containing less than 0 5 mM inorganic sulfate led to a decreased sulfate incorporation The use of potential sulfate decreasing drugs can lead to inhibition of glycosaminoglycan synthesis This argues for a reconsideration of the use of these drugs in patients with already dysfunctioning cartilage metabolism as in rheumatoid arthritis and osteoarthrosis

Nonsteroidal antiinflammatory drugs (NSAIDs) are used in the treatment of both rheumatoid arthritis and osteoarthrosis (osteoarthritis) These diseases are associated with the pathology of articular cartilage However in the case of osteoarthrosis cartilage destruction is a primary event while in rheumatoid arthritis it is a secondary phenomenon following the inflammation of synovial tissue Several investigations have demonstrated that certain NSAIDs might affect the biochemical properties of articular cartilage chondrocytes

NSAIDs can exert their pharmacological effects on chondrocytes in two ways a direct effect on the biochemical activities of chondrocytes and/or an indirect effect by way of influencing systemic factors determining the function of chondrocytes Many direct effects of NSAIDs are reported in the literature [1–7] Salicylates are reported by Palmoski and Brandt to suppress glycosaminoglycan synthesis both *in vitro* and *in vivo* [8–11] These authors showed that this effect was a direct effect of salicylate on the chondrocyte metabolism in their experiments [12] An indirect effect of salicylate on glycosaminoglycan synthesis in articular cartilage has been observed in our experiments [13, 14] A decrease of serum sulfate concentration in mice by way of salicylate induced sulfate diuresis apparently was the causative factor producing a diminished synthesis of glycosaminoglycans in anatomically intact patellar cartilage [13 14]

Herein we describe the effect of serum sulfate reduction on cartilage glycosaminoglycan synthesis in more detail We used paracetamol instead of salicylate to decrease sulfate availability In this way possible direct effects of salicylate on glycosamino glycan synthesis are eliminated Paracetamol is conjugated with sulfate by rat liver enzymes resulting in depletion of serum sulfate [15] We used in this study Wistar rats instead of mice because rats are relatively insensitive to paracetamol induced hepatoxicity and have a higher capacity of paracetamol sulfoconjugation than mice [16, 17]

MATERIALS AND METHODS

Effect of paracetamol on the serum sulfate concentration Male Wistar rats (150–200 g) were used in all experiments They were fed with a commercial pellet diet and given fresh tap water *ad libitum* Paracetamol (200 mg/kg) dissolved in tap water (1 ml) was orally administered to the rats Control animals received a comparable volume of only tap water After 2 5 7 and 24 hr a blood sample was taken by orbita-punction under a light ether anesthesia Inorganic sulfate was determined by a modification of the benzidine method of Dogson and Spencer [18] as recently described [19]

Effect of paracetamol and salicylate on glycosaminoglycan synthesis in vitro Wistar rats were killed by cervical dislocation and both intact patellae were with a standard amount of surrounding tissue dissected from the knee joints Patellae were incubated in RPMI 1640 DM medium supplemented with 2 mM L glutamine and 1 mM

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pyruvate (all from Flow Laboratories Irvine Scotland) The sulfate concentration in the medium was 0 6 mM Various concentrations of paracetamol and sodium salicylate (1 2 and 5 mM) were added to the medium at the initiation of the 4-hr incubation period Administration of 200 mg/kg paracetamol to male Wistar rats (150–200 g) as in our experiments will result in a maximum serum paracetamol concentration of 0 2–0 3 mM [15] After 2 hr incubation 20 μCi [^{35}S]sulfate (Radiochemical Centre Amersham U K) was added to the medium All incubations were performed at 37° in a humidified atmosphere containing 5% CO_2 Patellae were washed twice with physiological saline and subsequently fixed in ethanol (96% v/v) Decalcification of the patellae with 5% (v/v) formic acid was followed by stripping of the patellar cartilage layer [19] Patellar cartilage was digested overnight at 60° by solulyte (J T Baker Chemicals Deventer The Netherlands) and the amount of incorporated [^{35}S]sulfate per cartilage of one patella was assayed by liquid scintillation analysis

Effect of paracetamol on the catabolism of [^{35}S]sulfate labeled glycosaminoglycans Patellae were incubated in culture medium containing 80 μCi [^{35}S]sulfate/ml for 2 hr as described above This labeling period was followed by a chase without radiolabel for 16 hr in medium containing 0 1 2 or 5 mM paracetamol This was succeeded by processing of the patellae as described above

Effect of medium sulfate concentration on glycosaminoglycan synthesis in vitro Rat patellae were incubated in BMF diploid medium (Flow Laboratories) with various sulfate concentrations for 2 hr at 37° in humidified 5% CO_2 atmosphere The BMF medium was supplemented with 2 mM L-glutamine 1 mM pyruvate 20 μCi [^{35}S]sulfate (carrier free) and 40 μCi D [6 ^{3}H]glucosamine (RCA) per ml respectively Patellar cartilage was isolated from the surrounding tissue as described above and glycosaminoglycans were isolated To this end patellar cartilage was digested overnight by papain at 60° The papain mixture (pH 6 0) consisted of 1 mg/ml papain (type IV double crystallized Sigma St Louis MO U S A) 0 1 M Na-acetate 10 mM cysteine hydrochloride and 50 mM Na_2 EDTA Non hydrolysed remnants were spun down (10 000 *g* 15 min) and 150 μl 0 2% cetylpyridinium chloride (CPC) was added to 150 μl supernatant After 2 hr incubation at 37° the precipitate was centrifuged (10 000 *g* for 15 min) at 37° The supernatant was discarded and the pellet was washed twice with 0 5 ml 0 05% CPC The pellet was solubilized for 2 hr in 0 5 ml solulyte at 60° and after addition of scintillation fluid the quantity of radioactivity was analyzed with a liquid scintillation counter Appropriate corrections were made for channel overlap of ^{35}S counts and ^{3}H-counts

Effect of paracetamol on the glycosaminoglycan synthesis in vivo Two hours after oral administration of 200 mg/kg paracetamol or tap water to male Wistar rats 1 μCi/g [^{35}S]sulfate (carrier free) was injected intravenously (five rats per group) Blood samples were taken by orbita-punction at 20 min and 1 2 3 4 and 5 hr after injection of [^{35}S]sulfate Patellae were dissected from the knee joints 5 hr after administration of radiolabeled sulfate Patellae were processed for determination of the [^{35}S]sulfate content as described above

Total inorganic sulfate in serum of the rats was quantitated as described above To determine the percentage of the free inorganic sulfate form of [^{35}S] in the serum of the rats inorganic sulfate was precipitated with $BaCl_2$ To 50 μl of serum 1 ml of trichloroacetic acid (5% w/v) was added This mixture was allowed to stand at room temperature for 10 min and was subsequently centrifuged for 15 min (10 000 *g*) Five hundred microlitres of clear supernatant was added to 125 μl $BaCl_2$ reagent (20 g $BaCl_2$ $2H_2O$ and 100 g Dextran in 1 l distilled water) The precipitate was spun down (30 min 10 000 *g*) after overnight incubation at 4° The radioactivity in the pellet and supernatant was assayed by liquid scintillation counting At whatever time in the serum of control rats always more than 93% of total serum [^{35}S] was precipitated In the serum of the paracetamol treated rats 50–60% of the total [^{35}S] was precipitable

Characterization of glycosaminoglycans synthesized in vivo Tap water or 200 mg kg paracetamol was orally administrated to male Wistar rats (two rats per group) and after 2 hr a physiological saline solution with 1 μCi g [^{35}S]sulfate and 8 μCi g [^{3}H]glucosamine was injected intravenously Five hours after injection of radiolabel the patellae were dissected from the knee joints and articular cartilage was isolated as described above After papain digestion and spinning down of the undigested remnants the supernatants were dialysed for 72 hr against tap water This was followed by lyophilization and redissolving of the glycosaminoglycans in the elution buffers used during chromatography

Glycosaminoglycans of individual patellae were applied on a DEAE-Trisacryl anion exchange column (1 × 15 cm) equilibrated with 200 ml 0 2 M NaCl in 1 mM HCl A linear gradient of 0 2–2 0 M NaCl in 1 mM HCl was used to elute the glycosaminoglycans from the column After each run the column was washed with 100 ml 0 2 M NaCl in 1 mM HCl The flow was 20 ml hr and fractions of 2 ml were collected The fractions were assayed for radioactive labelled glycosaminoglycans by liquid scintillation counting

Glycosaminoglycans of individual patellae were also applied on a Sepharose CL 6B gel chromatography column (100 × 0 7 cm) An 0 1 M ammonium acetate buffer (pH 5 0) was used as eluent The flow was 15 ml hr and fractions of 1 25 ml were collected and assayed for radioactive labeled glycosaminoglycans The void volume (V_0) was determined by dextran blue (M 2×10^6) and the total volume (V_t) by Na_2[^{35}S]SO_4

Statistical analysis Statistical analysis of data was performed by the two tailed Student t test A P-value < 0 05 was considered significant

RESULTS

Decrease of serum sulfate level by paracetamol

Oral administration of 200 mg/kg paracetamol to Wistar rats led to a significant reduction of the inorganic sulfate concentration in serum (Fig 1) Five

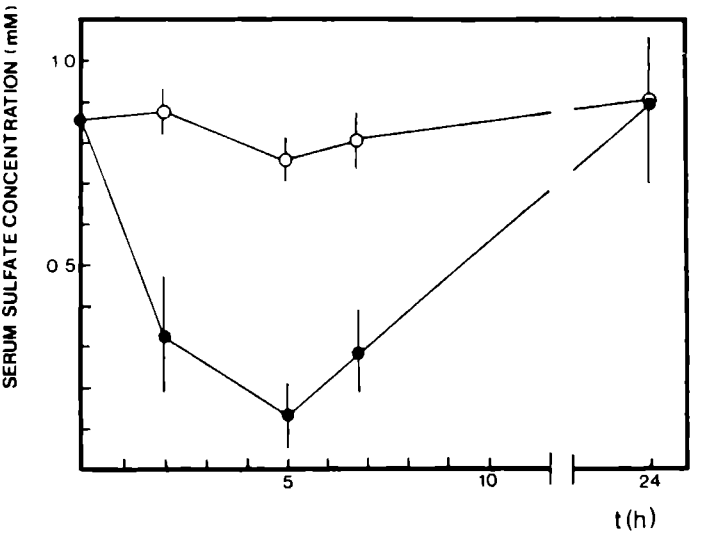

Fig 1 Effect of the oral administration of 200 mg/kg paracetamol on the serum sulfate concentration of male Wistar rats Control rats received a comparable volume of tap water The results are expressed as the mean ± SD of five rats (● paracetamol treated rats ○ control rats)

hours after paracetamol administration the serum sulfate concentration was decreased by more than 80% after 7 hr the sulfate depletion was still 66%

Effect of paracetamol and salicylate on [^{35}S]*sulfate labeled glycosaminoglycan synthesis* in vitro

In-vitro incubation of anatomically intact rat patellae with sodium salicylate resulted in a significantly decreased incorporation of [^{35}S]sulfate already at a concentration of 1 mM (Fig 2) Paracetamol had up to a concentration of 5 mM, the highest concentration tested, no effect on incorporation of radiolabeled sulfate (oral administration of 200 mg/kg will result in a peak serum level of approximately 0 2–0 3 mM [15]) The incubations were carried out in the absence of serum, so the added drug concentrations were equivalent with the free drug concentrations in the incubation media

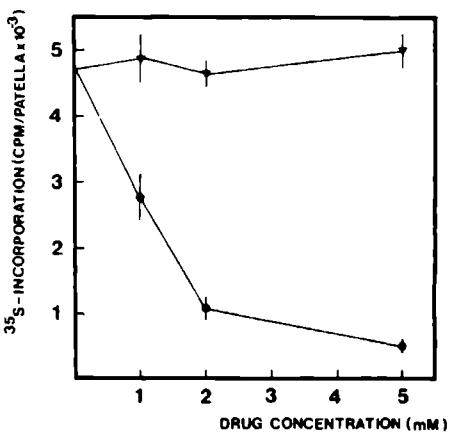

Fig 2 Effect of sodium salicylate and paracetamol on the incorporation of [^{35}S]sulfate in anatomically intact articular cartilage of rat patellae *in vitro* Patellae were incubated for 4 hr in medium containing sodium salicylate or paracetamol For the last 2 hr the patellae were incubated in the presence of 20 μCi [^{35}S]sulfate The results are expressed as the mean ± SD of five patellae (● salicylate, ▼ paracetamol)

Effect of paracetamol on the degradation of [^{35}S]*sulfate labeled glycosaminoglycans*

Paracetamol had a slight but significant inhibitory effect on the catabolism of radiolabeled glycosaminoglycans *in vitro* (Table 1) All three paracetamol concentrations tested had a significant suppressing effect on the breakdown of radiolabeled glycosaminoglycans but a dose response was not observed

Table 1 The effect of paracetamol on the degradation of [^{35}S]sulfate labeled glycosaminoglycans *in vitro*

Paracetamol (mM)	Chase (hr)	[^{35}S]sulfate content of patellae (cpm)
—	0	19 232 ± 3407
0	16	10 148 ± 878
1	16	13 040 ± 1153
2	16	13 864 ± 2768
5	16	11 777 ± 1427

Expressed are the mean values ± SD of at least five patellae A 2-hr labeling period was followed by a 16 hr chase period in culture medium without radiolabel

Effect of the sulfate concentration in medium on glycosaminoglycan synthesis

The incorporation of sulfate and glucosamine in the glycosaminoglycans of patellar cartilage incubated in medium with various sulfate concentrations is shown in Fig 3 The incorporated quantity of sulfate and glucosamine was calculated by the incorporation of radiolabeled sulfate and glucosamine corrected for the specific activities of these precursors in the various experimental conditions Incubation of patellae in medium containing less than 0 5 mM sulfate resulted in significantly reduced incorporation of sulfate in patellar cartilage glycosaminoglycans The incorporation of glucosamine in patellar cartilage glycosaminoglycans was not diminished at sulfate concentrations below 0 5 mM These results suggested the synthesis of undersulfated glycosaminoglycans at sulfate concentrations below 0 5 mM

Effect of serum sulfate depletion on glycosaminoglycan synthesis in vivo

The *in-vitro* experiments described above show that a reduction of the sulfate concentration leads to a diminished sulfate incorporation and indicates the synthesis of undersulfated glycosaminoglycans at low sulfate concentrations To answer the question if serum sulfate depletion leads to a diminished synthesis of glycosaminoglycans *in vivo* rats were injected with radiolabeled sulfate 2 hr after oral administration of 200 mg/kg paracetamol Patellae were dissected from the knee joints 5 hr after injection of radiolabel The specific activity of [^{35}S]sulfate in the serum of the paracetamol treated and control rats in this 5-hr period is shown in Fig 4 The area under the specific activity curve of the paracetamol treated rats was 1 52 greater than the area under the curve of the control rats Moreover the specific activity in the serum of the paracetamol treated rats decreased faster than the specific activity in the serum of the control rats

The incorporation of radiolabel in the patellar cartilage of the paracetamol treated rats and the control rats is shown in Table 2 Incorporation of sulfate in the patellar cartilage of serum sulfate depleted rats was significantly diminished (66%) The paracetamol-induced serum sulfate depletion leads to a decreased synthesis of cartilage glycosaminoglycans

Characterization of newly in vivo synthesized glycosaminoglycans

Our *in vitro* experiments indicated the synthesis of undersulfated glycosaminoglycans at low sulfate concentrations Therefore *in vivo* newly synthesized glycosaminoglycans were characterized by column chromatography to examine the presence of undersulfated glycosaminoglycans in the cartilage of the paracetamol treated rats DEAE-Trisacryl anion exchange analysis of patellar glycosaminoglycans synthesized *in vivo* by paracetamol treated and control rats resulted in the chromatograms shown in Fig 5 Both the chromatogram of the paracetamol treated and control rats showed the presence of one peak The peak in both chromatograms eluted at a NaCl concentration of 0 7 M In the chromatogram of the sulfate depleted rats (paracetamol treated) neither a shift of the sulfated glycosaminoglycan

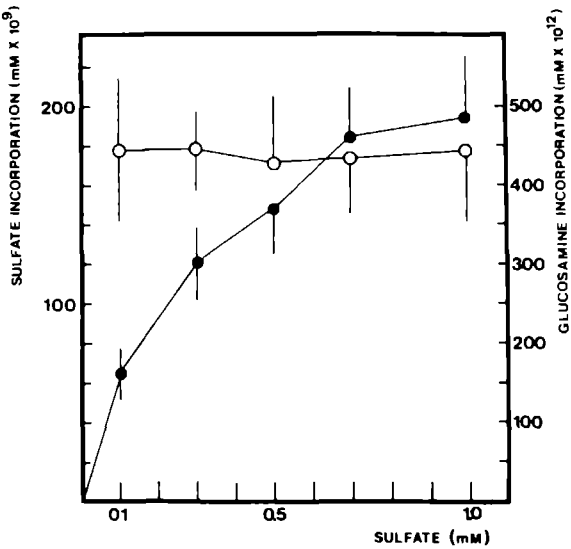

Fig 3 Effect of sulfate concentration in the medium on incorporation of sulfate and glucosamine in anatomically intact articular cartilage of rat patellae Patellae were incubated for 2 hr in BME diploid medium with various sulfate concentrations The results are expressed as the mean ± SD of at least five patellae (● sulfate ○ glucosamine)

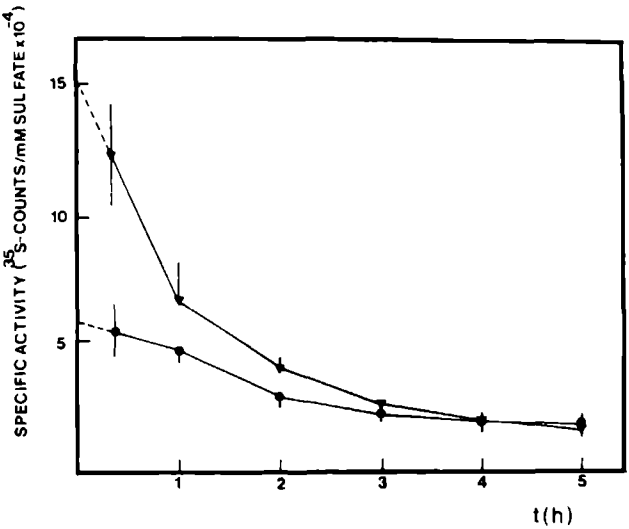

Fig 4 The specific activity of free inorganic [^{35}S]sulfate in the serum of paracetamol treated and control rats Paracetamol was orally administered 2 hr before injection of radiolabelled sulfate ($t = 0$) The results are expressed as the mean + SD of five rats (▼, paracetamol treated rats ● control rats)

peak nor the presence of a non-sulfated glycosaminoglycan peak was observed Also the length of the newly synthesized glycosaminoglycan chains, as determined by gel chromatography with Sepharose CL 6B, was similar for the sulfate depleted and the control rats (Fig 6) The K_{AV} of the glycosaminoglycan chains was 0 44

DISCUSSION

The oral administration of 200 mg/kg paracetamol to male Wistar rats resulted in a significant reduction of the serum sulfate concentration This serum sulfate depletion led to a diminished incorporation of [^{35}S]sulfate in patellar cartilage indicating a decreased glycosaminoglycan synthesis Also incubation of rat patellae in medium containing less than 0 5 mM sulfate reduced the incorporation of sulfate However the incorporation of glucosamine was unaltered in these experiments at low sulfate concentrations in the medium Characterization of patellar glycosaminoglycans synthesized *in vivo* could not confirm the production of undersulfated glycosaminoglycans as suggested by these results The use of sulfate decreasing drugs can lead to a diminished glycosaminoglycan synthesis but will not alter the biochemical nature of these glycosaminoglycans, at least in short term

In earlier studies we reported the inhibitory effect of salicylate induced sulfate depletion on glycosaminoglycan synthesis in mice [13, 14] Salicylate induced an elevated excretion of sulfate by the kidneys [13, 14] Incubation of murine cartilage in the presence of 5 mM salicylate inhibited sulfate incorporation 46% [13] while the incorporation in rat cartilage was inhibited by more than 90% (Fig 2) Rat chondrocytes are more vulnerable to salicylate than murine chondrocytes

The potential direct effects of salicylate on the metabolism of chondrocytes makes this drug less suitable for *in-vivo* studies on the effects of sulfate depletion on glycosaminoglycan synthesis [8] Phe-

Table 2 Incorporation of [^{35}S]sulfate in patellar cartilage of paracetamol treated rats and control rats

	[^{35}S]sulfate incorporation in patellar cartilage		
	Non-corrected value (cpm)	Corrected value (cpm)	Percentage of control
Paracetamol treated rats	6285 ± 692	4135 + 455	66
Control rats	6051 ± 550	6051 ± 550	100

Expressed are mean values ± SD of the patellae of five rats The values in the second column are corrected for the difference between the specific activity in the serum of the paracetamol treated rats and in the serum of the control rats (Fig 4 area under the curve ratio is 1 52)

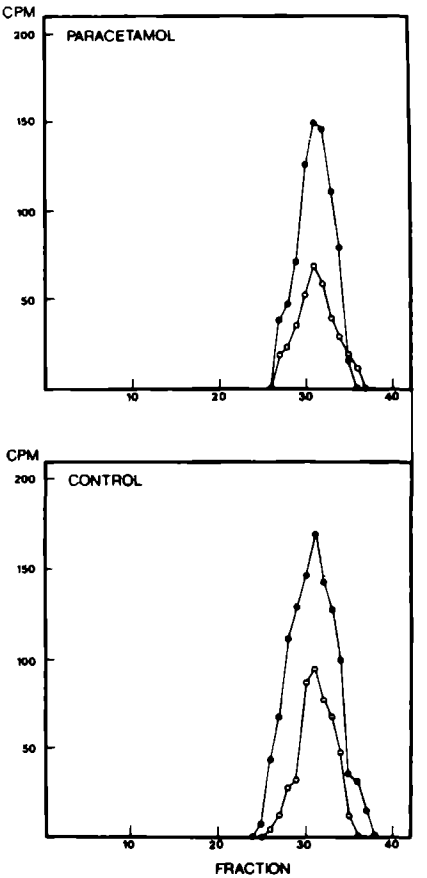

Fig 5 DEAE–Trisacryl anion exchange chromatogram of newly synthesized patellar cartilage glycosaminoglycans from paracetamol-treated and control rats Glycosaminoglycans were eluted with a NaCl gradient (0 2–2 0 M NaCl in 1 mM HCl) Fractions of 2 ml were collected and radioactivity was determined by liquid scintillation counting (●, [^{35}S]-counts, ○, [^{3}H] counts)

nolic compounds like paracetamol and salicylamide are known to decrease the inorganic serum sulfate level by conjugation with sulfate [15, 20, 21] Morris and Levy reported a significant reduction of the serum sulfate concentration in healthy human volunteers after a single dose of 1 5 g paracetamol [22]

Paracetamol (200 mg/kg) decreased the serum sulfate level in male Wistar rats significantly The oral administration of this dose of paracetamol to male Wistar rats will result in a maximal serum paracetamol concentration of 0 2–0 3 mM, 1 hr after administration [15] Paracetamol had no effect on the glycosaminoglycan synthesis up to the highest concentration tested (5 mM) while the glycosaminoglycan synthesis was already significantly inhibited by 1 mM salicylate (Fig 2) Paracetamol had, *in vitro*, a slight inhibitory effect on the degradation of [^{35}S]sulfate labeled glycosaminoglycans Paracetamol is a suitable drug to study the effect of sulfate depletion on glycosaminoglycan synthesis *in vivo* in the rat

This study showed that low sulfate concentrations resulted both *in vitro* and *in vivo* in a diminished incorporation of sulfate in anatomically intact articular cartilage of the rat Similar *in-vitro* results were found in experiments of Maroudas and Evans with slices of human and bovine cartilage and by Bayliss *et al* for rabbit annulus cartilage [23 24] Also *in-vitro* incubation of anatomically intact patellar cartilage of mice in medium with less than 0 5 mM sulfate led to a diminished sulfate incorporation [13]

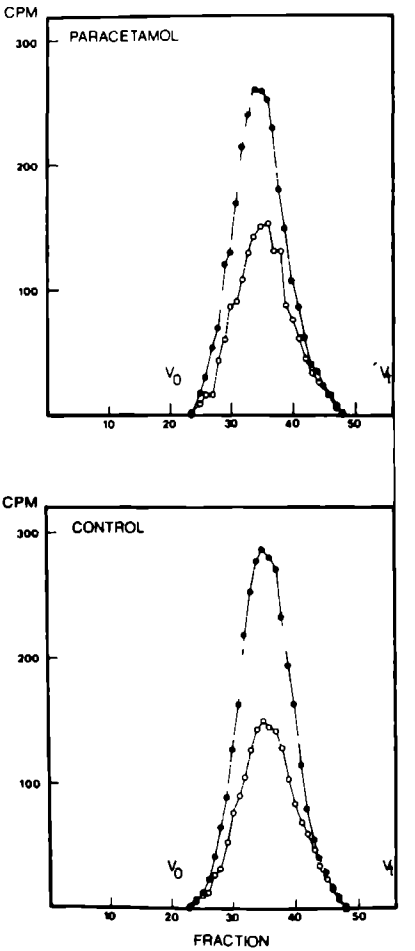

Fig 6 Sepharose CL 6B chromatogram of newly synthesized patellar cartilage glycosaminoglycans from paracetamol treated and control rats Glycosaminoglycans were eluted with an ammonium acetate buffer (0 1 M pH 5 0) Fractions of 1 25 ml were collected and radioactivity was assayed by liquid scintillation counting (● [^{35}S] counts, ○ [^{3}H] counts)

A decreased sulfate incorporation in cartilage is considered to be a reflection of a diminished glycosaminoglycan synthesis However the decreased sulfate incorporation in our *in-vitro* experiments did not show a concomitant decrease of glucosamine incorporation a precursor of the glycosaminoglycan backbone This suggested the synthesis of undersulfated glycosaminoglycans Seegmiller *et al* also found a decreased incorporation of sulfate and an unaltered incorporation of glucosamine after treatment of cartilage epiphyses from chick embryos with 6 aminonicotinamide [25] Characterization with anion exchange chromatography of patellar cartilage glycosaminoglycans synthesized *in vivo* at decreased sulfate concentrations could not confirm the synthesis of undersulfated glycosaminoglycans (Fig 5) Incubation of murine patellae *in vitro* at low sulfate concentrations (<0 5 mM) also resulted in decreased sulfate and unaltered glucosamine incorporation The glycosaminoglycans synthesized at a high sulfate concentration (1 mM) were similar to those synthesized at a low sulfate concentration (0 1 mM) (manuscript submitted)

The unaltered incorporation of glucosamine in the presence of a decreased glycosaminoglycan synthesis could be the result of changes in the intracellular specific activity of [H]glucosamine We postulate the hypothesis that at a high rate of glycosaminoglycan synthesis the demand for glucosamine will be high and the glucose component of glucosamine will be derived from the intracellular glycogen pool in the chondrocytes A decreased synthesis of glycosaminoglycans as a consequence of sulfate shortage or glycosaminoglycan synthesis inhibiting compounds will result in a reduced degradation of glycogen to glucose The reduced intracellular supply of glucose and consequently of unlabeled glucosamine will result in an increased specific activity of [^{3}H]glycosamine This could be an explanation for the apparent unaltered incorporation of glucosamine at low sulfate concentrations

In this study we demonstrate that reduction of the serum sulfate concentration can lead to inhibition of glycosaminoglycan synthesis *in vivo* The biochemical quantities of the glycosaminoglycans synthesized at low sulfate concentration were similar with those synthesized at physiological sulfate concentrations Conjugation with sulfate is a common physiological way of drug detoxification [15] Drugs can also effect the renal excretion of sulfate [13 14] Both mechanisms can lead to sulfate depletion and consequently to inhibition of glycosaminoglycan synthesis The glycosaminoglycan synthesis in man will be extremely sensitive for sulfate depletion because man has a very low serum sulfate level (0 3–0 4 mM) and sulfate concentrations just below 0 3 mM do already result in a diminished synthesis of glycosaminoglycans in human articular cartilage [22 23] The use of potential serum sulfate decreasing drugs might have deleterious effects on cartilage especially when used for prolonged periods This argues for a reconsideration of the use of potential sulfate decreasing drugs in patients with rheumatoid arthritis or osteoarthritis who already have a disturbed cartilage metabolism

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THE EFFECT OF CHRONIC PARACETAMOL ADMINISTRATION TO RATS ON THE GLYCOSAMINOGLYCAN CONTENT OF PATELLAR CARTILAGE

Peter M. van der Kraan, Elly L. Vitters, Bernard J. de Vries, Wim B. van den Berg and Levinus B.A. van de Putte

Agents and Actions: in press

ABSTRACT

Male Wistar rats were treated with paracetamol (200 mg\kg twice a day) for 2, 3, 4 and 9 weeks. During the first four weeks of paracetamol administration the serum sulfate concentration was significantly decreased. However, during the fourth until the ninth week, the serum sulfate concentration was only diminished to a small and insignificant extent. The paracetamol administration did not lead to serious liver or renal toxicity, as determined by alanine aminotransferase and creatinine levels in the serum of the rats.

The paracetamol-induced serum sulfate depletion, observed during the first four weeks of the experiment, led to a significantly lower glycosaminoglycan content of the patellar cartilage of the rats after three and four weeks paracetamol treatment. When after the fourth week the serum sulfate concentration rose to nearly normal levels also the glycosaminoglycan content in the rat patellar cartilage reached control levels. These data indicate that the serum sulfate depletion might be the causative factor for the observed reduction in glycosaminoglycan content of rat patellar cartilage.

Both rheumatoid arthritis and osteoarthritis are diseases associated with pathology of articular cartilage. In the case of osteoarthritis this is a primary phenomenon while in rheumatoid arthritis it is an event secondary to inflammation of the synovial tissue. In the treatment of both diseases nonsteroidal antiinflammatory drugs (NSAIDs) are frequently used for their analgetic and antiinflammatory properties. However, numerous investigations have demonstrated that certain NSAIDs might affect the metabolism of articular cartilage chondrocytes.

There are essentially two ways by which NSAIDs can alter the metabolism of articular cartilage chondrocytes: A direct effect of NSAIDs on the biochemical activities of chondrocytes and/or an indirect effect on chondrocyte metabolism by way of influencing systemic factors. Several investigators have reported the drug-induced inhibition of chondrocyte glycosaminoglycan synthesis by way of the first mechanism [1,2,3,4]. Also, well known is the suppressive effect of salicylate on glycosaminoglycan synthesis of cartilage both in vitro and in vivo experiments [5,6,7,8]. This was claimed to be a direct effect of salicylate on chondrocyte metabolism.

We observed that salicylate has also an indirect effect on the glycosaminoglycan synthesis in articular cartilage [9,10]. A diminished serum sulfate concentration due to salicylate-induced sulfate depletion was held responsible for a decreased synthesis of glycosaminoglycans in anatomically intact patellar cartilage of the mouse [9,10].

In recent studies we have used paracetamol, instead of salicylate, to decrease the sulfate availability in male Wistar rats. Paracetamol is conjugated with sulfate by rat liver enzymes resulting in depletion of serum sulfate [11]. Paracetamol had, in contrast to salicylate, no direct inhibitory effect on chondrocyte metabolism [12]. In previous experiments we observed that a single oral dose of paracetamol (200 mg/kg) diminished the serum sulfate concentration in male Wistar rats from 0.8 mM to nearly 0.1 mM in five hours [12,13]. This eventually resulted in a 34% inhibition of glycosaminoglycan synthesis in rat patellar cartilage [12]. We describe the effects of long term paracetamol administration to

male Wistar rats on the glycosaminoglycan content of patellar cartilage.

METHODS

Paracetamol administration

Male Wistar rats (190-210 g) were used in all experiments. They were fed a commercial pellet diet (RHM, Hope farms, Linschoten, The Netherlands) and given fresh tap water ad libitum. Paracetamol was orally administrated twice a day in a dose of 200 mg/kg on 9.00 a.m. and 5.00 p.m. from monday through friday (1.5 ml). In the weekends they received one dose a day on 11.00 a.m.. To facilitate drug administration, paracetamol was suspended in a solution of 0.1 % methylcellulose. Control animals received a comparable volume of only the methylcellulose solution.

Effect of paracetamol on serum sulfate concentrations

Once a week (wednesday 2.00 p.m.) blood samples were taken by orbita-punction under a light ether anesthesia and thereafter the rats were weighed. The inorganic sulfate concentration was determined by a modification of the benzidine method as recently described [14].

Alanine aminotransferase assay

Although rats are relatively insensitive to paracetamol-induced hepatotoxicity compared to other laboratory animals or man [15,16,17,18], we have measured the serum alanine aminotransferase levels at the moment that both the paracetamol-treated and the control rats were sacrificed (after 2, 3, 4 and 9 weeks). The alanine aminotransferase activity in serum was determined by the method of Ellis et al. [19].

Creatinine assay

Reduced or absent renal function is associated with pronounced retention of inorganic sulfate. A strong positive correlation is

observed between the serum concentrations of creatinine and inorganic sulfate in humans and rats with renal dysfunction [20,21]. Therefore we examined the effects of chronic paracetamol-treatment on serum creatinine levels. Just following sacrification, after 2, 3, 4 and 9 weeks, blood was obtained from the paracetamol-treated and the control rats. The quantity of serum creatinine was determined by the method of Bartels et al. [22].

Determination of the patellar glycosaminoglycan content

After 2, 3, 4 and 9 weeks rats were killed by cervical dislocation and the intact patellae were dissected from the surrounding tissue of the knee joint. The patellae were fixed in ethanol (96% v/v) for 24 hours and subsequently decalcified in formic acid (5% v/v) overnight. The cartilage layer was stripped off [14] and digested overnight at 60 °C by papain (type IV, double crystallized, Sigma, St Louis, MO). Following the papain digestion non-hydrolized remnants were spun down (1000 x g, 10 min). The quantity of glycosaminoglycans in the supernatant was determined by the dimethylmethelene blue assay described by Farndale [23]. Chondroitin-4-sulfate (Whale, Sigma) was used as a standard.

Statistical evaluation

Statistical evaluation of the results was done by analysis of variance (F-test). A P value < 0.05 was considered to be significant.

RESULTS

Effect of paracetamol on the serum sulfate concentration

The oral administration of paracetamol resulted in a decreased sulfate concentration in serum of the Wistar rats, when measured five hours after the first daily dose (200 mg/kg) (figure 1). Only during the first three weeks of paracetamol treatment the serum sulfate concentration was diminished significantly to approximately 30-40% of the control levels. After four weeks the

decrease of the serum sulfate concentration was not significant anymore and only about 10-20%. During the entire experiment the serum sulfate concentration in the paracetamol treated rats was similar to the concentration in the control rats when determined before the first paracetamol administration at 9.00 a.m. (data not shown). The above described results show that in the first three to four weeks of the experiment the serum sulfate concentration was significantly depressed in the paracetamol treated rats during a major part of the day.

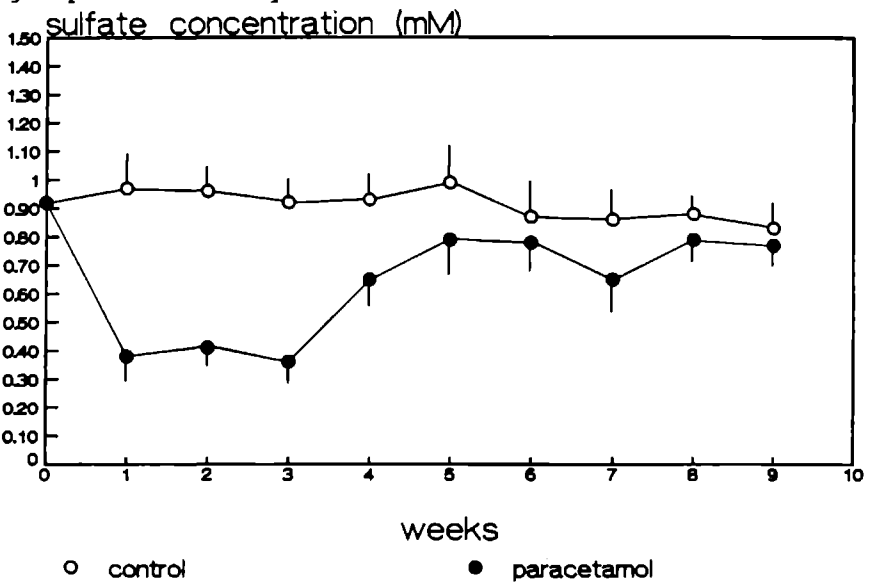

Fig 1. The effect of chronic paracetamol administration on serum sulfate concentration of male Wistar rats. Paracetamol was twice a day orally administrated in a dose of 200 mg\kg at 9.00 a.m. and 5.00 p.m. from monday through friday. In the weekends the rats received one dose a day at 11.00 a.m. Blood was collected on wednesday at 2.00 p.m.. The values are expressed as the mean ± s.d. of eight rats.

Effect of paracetamol on the growth of male Wistar rats

The control rats showed nearly linear growth during the nine weeks of the experiment, resulting in a weight gain of approximately 210 g (figure 2). The growth of the paracetamol-treated rats seemed to be slightly retarded. Nonetheless, the weight of the paracetamol-

treated and the control rats was not significantly different at any stage of the experiment.

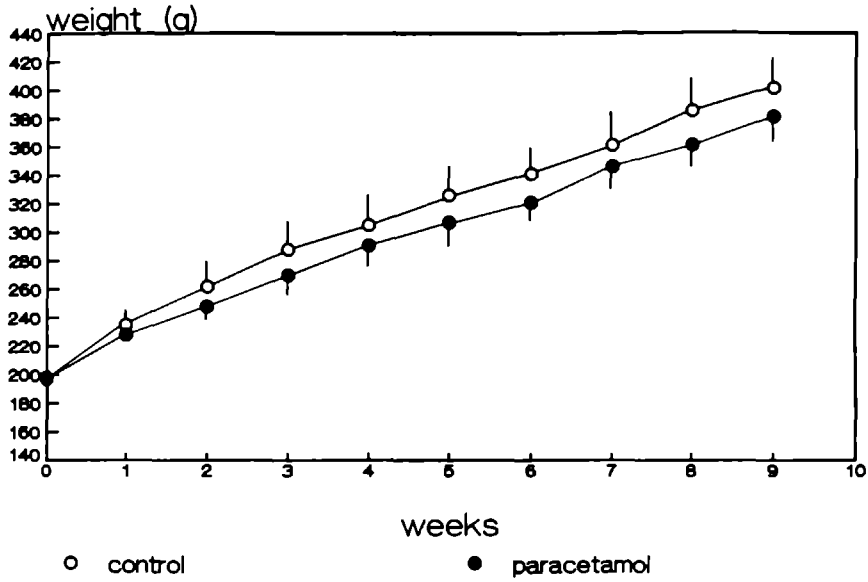

Fig 2. The effect of chronic paracetamol administration on the growth of male Wistar rats. The administration schedule is described in fig. 1. The rats were weighed once a week on wednesday at 2.00 p.m.. The values are expressed as the mean ± s.d. of eight rats.

Effect of paracetamol on the patellar glycosaminoglycan content

The effect of chronic paracetamol treatment on the glycosaminoglycan content of rat patellar cartilage is presented in table 1. Twice a day administration of paracetamol to Wistar rats (200 mg/kg) resulted after two weeks in a 6% less glycosaminoglycan content in the patellar cartilage (not significant). After three and four weeks respectively, the quantity of glycosaminoglycans was 8% and 12% lower in the cartilage of the paracetamol treated rats than in the cartilage of the control rats (significant). There was a significant increase in patellar glycosaminoglycan content from the fourth to the ninth week in the paracetamol-treated rats ($p<0.05$). A non significant reduction of only 2% was observed between the control and the

paracetamol-treated rats after nine weeks.

Table 1: The effect of chronic paracetamol administration (200 mg/kg twice a day) to male Wistar rats on the glycosaminoglycan content of patellar cartilage. The glycosaminoglycan content is expressed as the mean ± s.d. (μg/patella) of sixteen patellae.

paracetamol administration (weeks)	Glycosaminoglycan content control (μg)	paracetamol (μg)	
2	57.1 ± 6.7	53.4 ± 5.3	N.S.
3	57.6 ± 3.6	52.4 ± 5.3	P<0.05
4	58.5 ± 5.9	51.5 ± 1.7	P<0.01
9	60.8 ± 6.3	59.4 ± 6.7	N.S.

Effect of paracetamol on the serum alanine aminotransferase and creatinine levels

Paracetamol administration for as long as nine weeks twice a day had no effect on the alanine aminotransferase activity in serum of the rats (table 2), indicating that the chronic paracetamol administration did not induce serious hepatotoxicity in our experiments. A reduction of ALAT activity was observed from the second to the ninth week in the serum of the paracetamol-treated and the control rats.

The creatinine levels in the serum of the paracetamol-treated and the control rats are expressed in table 3. Significant differences between the paracetamol-treated and the control rats were not observed during the nine weeks of the experiments. This indicates that the chronic paracetamol administration to the rats had no effects on the renal function, as determined by serum creatinine levels, in this experiment. Both the control rats as well as the paracetamol-treated rats showed a reduction in creatinine levels during the experiment.

DISCUSSION

Paracetamol is widely used as an analgetic in the treatment of

both osteoarthritis and rheumatoid arthritis. In an earlier study we reported the effect of a single oral dose paracetamol (200 mg/kg) on the glycosaminoglycan synthesis in rat patellar cartilage [12]. A decrease in the serum sulfate concentration caused by paracetamol, appeared to be the causative factor for a 34% suppressed glycosaminoglycan synthesis in rat patellar cartilage in a short term study. The sulfate depletion had no effect on the degree of sulfation or on the length of the glycosaminoglycans synthesized [12].

Table 2: The effect of chronic paracetamol administration (200 mg/kg twice a day) on the alanine aminotransferase (ALAT) activity in the serum of male Wistar rats. The values are expressed as the mean ± s.d. of eight rats.

	AlAT activity		
paracetamol administration (weeks)	control (units)	paracetamol (units)	
2	39.6 ± 3.4	43.5 ± 5.3	N.S.
3	39.9 ± 4.3	42.4 ± 4.3	N.S.
4	34.9 ± 4.0	38.5 ± 9.9	N.S.
9	35.0 ± 7.6	30.3 ± 3.6	N.S.

Long term paracetamol administration led to a significant serum sulfate depletion during the first three weeks. However after four weeks the effect of paracetamol on the serum sulfate levels was greatly reduced (figure 1). This unexpected phenomenon did not seem to be caused by paracetamol-induced liver injury since the alanine aminotransferase levels in the serum of the paracetamol-treated rats were not elevated during the experiment (table 2). Also paracetamol-induced renal dysfunction is not a likely cause for the nearly normalized serum sulfate levels after four weeks, since the serum creatinine levels were not elevated during the same period, indicating normal sulfate excretion [20,21].
Hendrix-Treacy and co-workers have studied the rate of paracetamol elimination and the pattern of paracetamol metabolism after single or multiple dose of the drug to humans [24]. Both a single (650 mg) or multiple (5 x 650 mg, in a 30 hour period) administration

of paracetamol resulted in decreased serum sulfate levels in healthy human volunteers [24]. The fraction of paracetamol recovered in the urine as the sulfate conjugate was less and the glucuronide conjugate greater after multiple dosing than after a single paracetamol dose. This is probably caused by the reduced availability of sulfate after multiple dosing [24]. In contrast, patients on chronic paracetamol medication showed serum sulfate levels even higher than the control population. Despite of the high serum sulfate levels, the percentage of paracetamol excreted as the sulfate conjugate was much less than after single or multiple drug dosing [24]. These observations are similar to the observations in rats in the herein described experiment. Paracetamol administration in a short period leads to serum sulfate depletion while prolonged paracetamol administration lacks this effect. The lack of serum sulfate reduction by paracetamol after chronic treatment might be caused by changes in the ratio paracetamol sulfation/paracetamol glucuronidation in the liver, induced by the prolonged paracetamol treatment.

Table 3: The effect of chronic paracetamol administration (200 mg/kg twice a day) on the creatinine concentration in serum of male Wistar rats. The values are expressed as the mean ± s.d. (μM) of eight rats.

	Creatinine concentration		
Paracetamol administration (weeks)	control (μM)	paracetamol (μM)	
2	59.7 ± 8.7	54.8 ± 3.8	N.S.
3	48.4 ± 3.4	47.5 ± 1.4	N.S.
4	40.0 ± 5.4	42.4 ± 3.7	N.S.
9	38.6 ± 1.7	39.5 ± 2.9	N.S.

Hepatotoxicity associated with an overdose of paracetamol is well known in man and certain laboratory animals [16,17,18,25]. Theoretically, a diminished liver function could affect cartilage metabolism as the liver is the main organ for the synthesis of the

chondrocyte growth factor, insulin-like growth factor 1 (IGF-1) [26]. Liver damage can potentially lead to a diminished production of IGF-1 and by this way to a decreased glycosaminoglycan synthesis by articular chondrocytes. However, rats are relatively insensitive to the toxic effects of paracetamol [15,16,17,18] as confirmed by our experiments. Chronic paracetamol administration for 9 weeks, twice a day 200 mg\kg, did not result in hepatotoxicity as expressed in elevated serum alanine aminotransferase levels.

The serum sulfate level was significantly depressed until the fourth week of the experiment and raised thereafter to nearly control levels. The fall and rise of the sulfate level in serum appeared to be concomitant with the reduced (3 and 4 weeks) and nearly normalized glycosaminoglycan content in rat patellar cartilage (9 weeks). So, the serum sulfate level seems to be the causative factor for the diminished glycosaminoglycan content in the patellar cartilage of 3-4 weeks paracetamol treated rats.

Short term (3 weeks) paracetamol administration leads to depletion of serum sulfate and glycosaminoglycan content in rat patellar cartilage which is overcome when the paracetamol administration is continued. Similar effects of paracetamol on sulfate concentration are described in humans [24]. However, it has to be determined whether other potentially sulfate decreasing drugs may have similar effects on serum sulfate concentrations. Other sulfate decreasing drugs might not exhibit a reversible effect on sulfate pool upon long term adminstration. A more severe effect on glycosaminoglycan content of cartilage is expected under conditions of prolonged serum sulfate reduction.

Acknowledgements

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CHAPTER 7

DEGENERATIVE KNEE JOINT LESIONS IN MICE AFTER A SINGLE INTRA-ARTICULAR COLLAGENASE INJECTION. A NEW MODEL OF OSTEOARTHRITIS

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ABSTRACT

A single intra-articular injection with bacterial collagenase in the right knee joints of 10 weeks old male C57bl10 mice led to osteoarthritic lesions within a few weeks in these joints. The collagenase-induced osteoarthritis was characterized by severe degenerative cartilage lesions on the medial side of the femorotibial joint associated with patellar dislocation to the medial side of the joint, sclerosis of subchondral bone below the cartilage erosions, osteophyte formation and consequently deformity of the knee joints. The osteoarthritic alterations in the collagenase model closely resembled the changes observed in spontaneous osteoarthritis in aged mice. The intra-articular injection with collagenase probably results in damage to collagen type I containing joint structures, such as tendons, ligaments and menisci, leading to an instable knee joint that results in the osteoarthritic joint lesions observed in this model. The collagenase-induced osteoarthritis model offers the possibility to study experimental osteoarthritis in large animal groups of inbred strains within a restricted time span at low costs.

Osteoarthritis is a common affliction of human joints characterized by fibrillation, erosion of articular cartilage, proliferation of normally non-dividing chondrocytes, formation of osteophytes at joint margins, and sclerosis of subchondral bone. The etiopathogenesis of osteoarthritis is multifactorial, various forms of osteoarthritis can be characterized as being the consequence of abnormal mechanical stress, or acquired or congenital abnormalities of articular cartilage. In all cases, the final common pathway is articular cartilage degeneration.

Both spontaneous and induced osteoarthritis models in laboratory animals have been described. The incidence of spontaneous osteoarthritis in various mouse strains has been studied by Sokoloff (Sokoloff (1956), Sokoloff & Jay (1956)). A great variation in the development of osteoarthritis in the different mouse strains was observed. After 17 months of age, 93% of the STR/IN mice had osteoarthritis while this was only 2% in the A/LN strain (Sokoloff (1956), Sokoloff anfd Jay (1956)). Spontaneous articular cartilage degeneration in the medial site of the tibial plateau appears to be the initial event in the osteoarthritis prone STR/IN mice (Schunke et al. (1988)). In a closely related strain, the STR/ORT strain, spontaneous patella dislocation has been found to be associated with osteoarthritis of the knee joint (Walton (1977 a ,b and c, 1979)). Spontaneous osteoarthritis has been found in laboratory animals other than mice. For example, a high incidence of osteoarthritic lesions was observed in the 12-18 months age group of Hartley strain guinea pigs (Bendele & Hulman (1988)).

Induction of osteoarthritis in animal knee joints has mainly been carried out in two different ways. 1. Surgical damage to joint structures, like tendons and ligaments, resulting in instable joints and eventually osteoarthritis (Bendele (1987), Colombo et al (1983), Cooke & Chir (1986), McDevitt & Muir (1977), Moskowitz et al (1973), Pond & Nuki (1973), Schwartz et al. (1981)). 2. Intra-articular injection of chemicals interfering with cartilage metabolism (Bentley (1971), Goldenberg et al. (1982), Havdrup & Telhag (1977), Williams & Brandt (1984)).

In the new model described herein, we have used intra-articular injection of bacterial collagenase to induce osteoarthritis in

young C57Bl mouse knee joints. Intra-articular injection of collagenase was shown to have minimal direct effects on joint cartilage but seems to result in an instable joint by way of damaging other joint structures (ligaments, menisci). This eventually leads to osteoarthritic alterations in these joints closely resembling spontaneous osteoarthritis in C57Bl and other mouse strains (Schünke et al. (1988), Sokoloff (1956), Sokoloff & Jay (1956), Walton (1977 a, b and c, 1979)).

MATERIAL AND METHODS

Ten weeks old, male C57Bl10 mice were used in all experiments. The animals were kept in boxes with a sawdust bottom in an air conditioned room at constant temperature and were fed a standard laboratory diet (Hope Farms, Deventer, The Netherlands) with access to tap water ad libitum.

The right knee joint of the mice was injected once (microlange needle, 30 G1/2 L.B.,0.3 X 13 BL, Becton Dickinson, Dublin, Ireland), intra-articularly through the patellar ligament, with a 6 microliter solution of bacterial collagenase (248 u/mg, Worthington Biochemical Corporation, Freehold, New Jersey). Stretching of the hind-leg facilitates the intra-articular injection. Before injection the collagenase solution was filtered through a 0.2 um bacteria filter. The left control knee joint was injected with a similar volume of physiological saline. Three collagenase concentrations were used in the experiments namely 0.5% (w/v), 1.0% (w/v) and 1.5% (w/v).

After 1, 3, 7, 21 and 42 days groups of five mice per collagenase concentration were killed by cervical dislocation. Carefully dissected knee joints were fixed in phosphate-buffered formalin (pH 7.4) for five days and subsequently decalcified in 5% formic acid for four days. Standard processing of the tissue in a automatic tissue processing apparatus was followed by embedding of the knee joints in paraffin wax [11, 24]. Total frontal joint section were prepared (6 um) and stained with safranin O and fast green. Osteoarthritic changes in the joints were evaluated with a histological score ranging from 0 (no alterations) to + (noticeable pathological alterations) to ++ (markedly pathological alterations).

Three collagenase concentrations were used to induce osteoarthritic changes in the right knee joint of C57Bl10 mice. The alterations observed in the joints after the injection of 0.5%, 1.0% and 1.5% were essentially similar. However, the lesions induced by 1% and 1.5% collagenase were more pronounced than the lesions observed after injection of 0.5% collagenase. Differences in the effect of 1% or 1.5% collagenase were not observed. Only the results of the 1% collagenase injection will be presented. In table 1 an overview of the osteoarthritic alterations is shown.

Day 1

One day after the intra-articular injection of collagenase a medial dislocation of the patella could be observed in four out of five animals injected with 1% collagenase (figure 1A). Dislocation of the patella was never observed in the control (left) knee joints.

Patellar cartilage and cartilage of the facies patellaris femoris showed a moderate loss of safranin O staining, indicating a reduced proteoglycan concentration in these areas compared to controls (figure 1B). The cartilage of the tibial plateau or the femoral condyles was normally stained when compared to control knee joints. Changes on the surface of the articular cartilage were neither observed in the femoropatellar joint nor in the femorotibial joint in both collagenase and saline injected joints. The structure of the cruciate ligaments appeared to be disorganized by the collagenase injection (polarized light examination).

Signs of inflammation were also observed in the collagenase injected knee joints (figure 1B). Synovial infiltration of polymorphonuclear leukocytes and exudation of inflammatory cells in the synovial cavity was found. This was most marked in the area of the femoropatellar joint.

Day 3

Medial dislocation of the patella was evident in four of the five mice. Depletion of safranin O staining was greatly reduced in the cartilage of the femoropatellar joint compared to the depletion one day after injection. While depletion of safranin O staining

Table 1: Histologic evaluation of the osteoarthritic alterations in the collagenase injected (1%) right knee joints of individual male C57Bl10 mice (n=5). (0 = no alterations, + = noticeable pathological alterations, ++ = markedly pathological alterations).

	score	day 1	day 3	day 7	day 21	day 42
Patellar dislocation	0	1	1	1	1	1
	+	0	0	0	0	0
	++	4	4	4	4	4
Cartilage fibrillation	0	5	5	2	0	0
	+	0	0	3	0	0
	++	0	0	0	5	5
Erosion of non-calcified cartilage	0	5	5	5	0	0
	+	0	0	0	1	0
	++	0	0	0	4	5
Erosion of calcified cartilage	0	5	5	5	2	0
	+	0	0	0	3	0
	++	0	0	0	0	5
Erosion of bone	0	5	5	5	5	0
	+	0	0	0	0	1
	++	0	0	0	0	4
Chondrocyte loss	0	5	5	5	0	0
	+	0	0	0	1	0
	++	0	0	0	4	5
Osteophyte formation	0	5	5	5	0	0
	+	0	0	0	1	0
	++	0	0	0	4	5
Loss of Safranin O staining, femoropatellar	0	0	4	5	5	1
	+	5	1	0	0	4
	++	0	0	0	0	0
femorotibial	0	5	2	1	0	0
	+	0	3	4	0	0
	++	0	0	0	5	5
cruciate ligament damage	0	0	0	0	0	0
	+	5	5	5	1	0
	++	0	0	0	4	5
chondrocyte clusters	0	5	5	5	2	1
	+	0	0	0	3	4
	++	0	0	0	0	0
Synovial infiltration	0	0	0	0	0	0
	+	5	5	5	5	5
	++	0	0	0	0	0
Inflammatory exudation	0	0	2	4	4	4
	+	4	3	1	1	1
	++	1	0	0	0	0
Synovium hyperplasie	0	5	5	0	0	0
	+	0	0	2	0	0
	++	0	0	3	5	5

was overcome in the femoropatellar joint after three days, a slight reduction in the safranin O staining of the femorotibial cartilage was found three days after collagenase injection but not one day after this injection.
The inflammation of the joints on day three was markedly reduced when compared to the inflammation on day one. Infiltration of inflammatory cells in the synovium was reduced and only a few inflammatory cells could still be seen in the joint cavities.

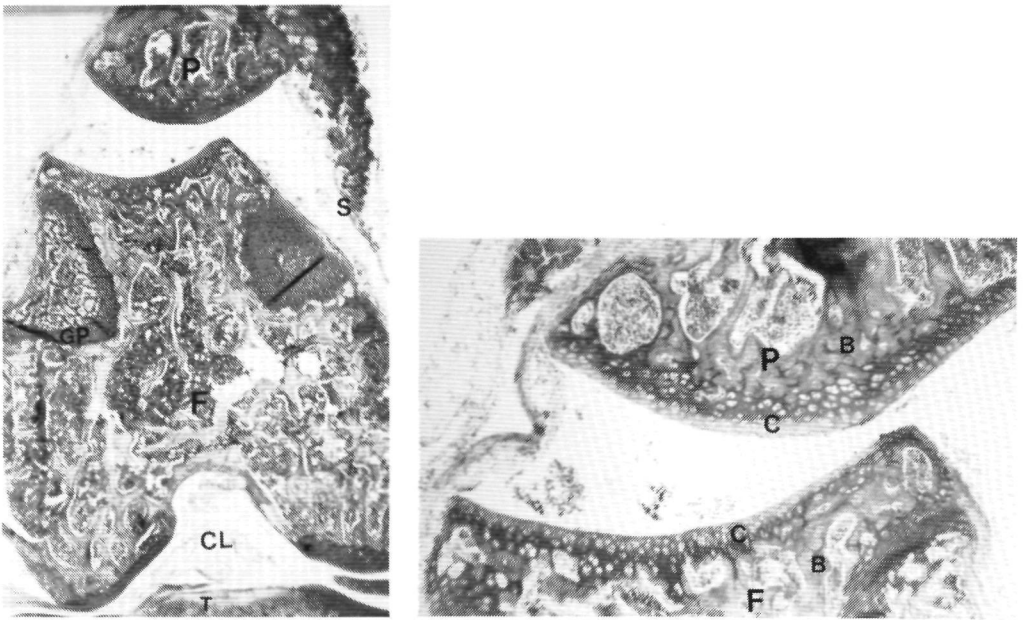

Figure 1: Right knee joint of a male C57Bl10 mouse one day after injection of 1% collagenase. A. Photograph of femoropatellar joint showing patella dislocation to the medial side of the joint (original magnification X 40). B. Photograph of femoropatellar joint showing loss of safranin O staining, patellar displacement and the presence of some inflammatory cells (original magnification X 100).P = patella, F = femur, GP = growth plate, C = cartilage, B = bone, T = tibia, Cl = cruciate ligaments, S = synovium, O = osteophyte.

Day 7
The safranin O staining of the cartilage of the femoropatellar joint was not reduced anymore but the cartilage of the

femorotibial joint was less stainable by safranin O than the control joints. Also initial fibrillation of cartilage was seen in these areas.
The synovial tissue on the medial side was thickened, indicating synovial cell proliferation (figure 2). Also the synovium on the lateral side was slightly thickened in some animals. Infiltration of inflammatory cells was not observed in the proliferated synovium but inflammatory cells could be seen in the original synovial lining.

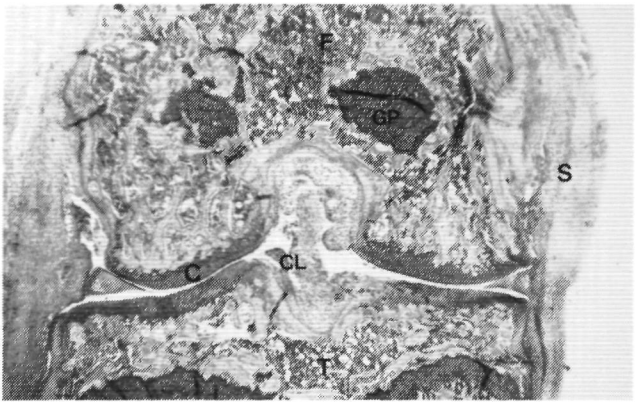

Figure 2: Right knee joint of a male C57Bl10 mouse seven days after injection of 1% collagenase. Hyperplasia of the synovium can be observed but infiltration of inflammatory cells was absent in the proliferated areas (Original magnification X 40). See also legend figure 1.

day 21
The animals show pronounced depletion of safranin O staining in the cartilage of the femorotibial joint 21 days after collagenase injection. This was most severe on the medial side of the tibial plateau and femoral condyle. The cell number in the proteoglycan-depleted cartilage was reduced but the cells still present in the depleted matrix were characterized by a highly safranin O stainable pericellular matrix (figure 3).
The cartilage on the medial side of the femoropatellar joint showed signs of degeneration. Fibrillation, clefts and eburnation of cartilage resulting in exposed bone was evident in this area (figure 4). Sometimes small chondrocyte clusters could be observed

in the affected cartilage. Degeneration of cartilage was also observed on the lateral side of the femoropatellar joint but this was always less severe than the alterations on the medial side. Osteoarthritic alterations in the femoropatellar joint were rare. There was a pronounced thickening of the medial synovium after injection of collagenase. On the lateral side the incidence was lower and the thickening less. Osteophyte formation could be seen on the medial side of the femoropatellar joint. The osteophytes were largest at the femoral condyles but also the tibial plateau showed pronounced osteophyte formation. Small osteophytes were present on the medial side of the femoropatellar joint in a few animals (figure 5). Hyperplasic changes in the menisci became evident 21 days after collagenase injection, and clearly enlarged ossific centre in the menisci could be seen.

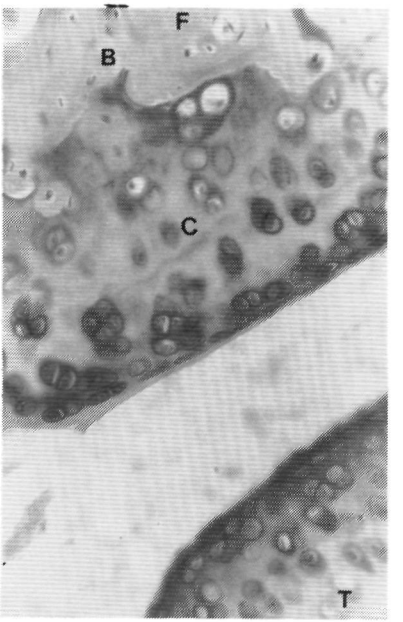

Figure 3: Cartilage of the femorotibial joint 21 days after injection of 1% collagenase. The chondrocytes present in the proteoglycan depleted matrix, as indicated by loss of safranin O staining, show a highly safranin O stainable pericellular matrix (original magnification X 400). See also legend figure 1.

Day 42

Forty-two days after collagenase injection the incidence of patellar dislocation was five out of five. The gross appearance of the affected joints was dramatically changed. Due to erosion of cartilage and bone and to osteophyte formation and synovial thickening the width of the knee joint was increased while the height of the joint appeared to be diminished in the knee joint sections compared to normal joints.

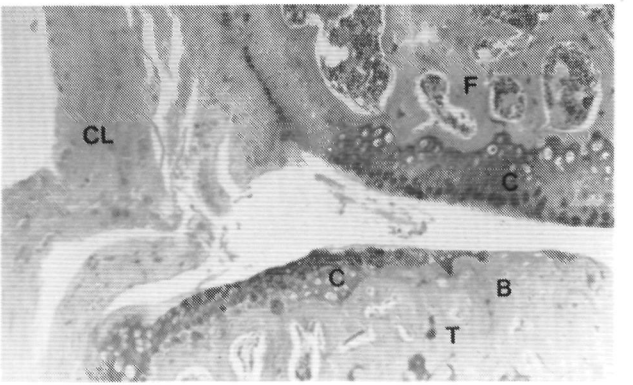

Figure 4: Medial side of the right femorotibial joint 21 days after injection of 1% collagenase. Fibrillation, erosion of non-calcified and calcified cartilage and sclerosis of subchondral bone can be observed (original magnification X 400). See also legend figure 1.

The osteoarthritic lesions in the knee joints injected with collagenase were severe. The cartilage of the medial side of the tibial plateau was totally eroded and in three of the five mice in this group the growth plate cartilage was exposed to the surface (figure 6). Below the cartilage and bone erosions, sclerosis of the remaining bone could be seen. Cartilage particles, apparently derived from the eroded sides, were present in the joint cavity. The lesions on the lateral side were less severe. The cartilage still present on the lateral side showed depletion of proteoglycans, as determined by loss of safranin O staining, and fibrillation and clefts. Small chondrocyte clusters, the pericellular matrix heavily stained by safranin O, could be seen

in the cartilage adjacent to the clefts (figure 7).
The tibial collateral ligament in the joint showed cartilaginous changes (figure 8) and ossification processes could be seen in the menisci. Osteophyte formation was advanced in the femerotibial joints and most severe on the medial side of the femorotibial joint. Small osteophytes on the medial side of the femoropatellar joint were also present in two out of five animals.

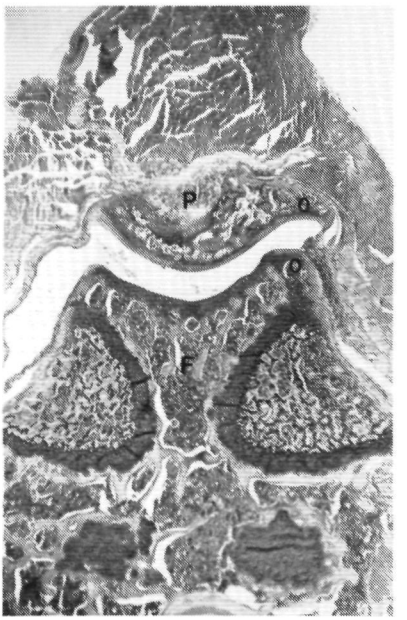

Figure 5: Femoropatellar joint of a right knee 21 days after injection of 1% collagenase. Osteophytes can be seen on the medial side of the femorotibial joint (original magnification X 40). See also legend figure 1.

DISCUSSION

A single intra-articular injection of C57Bl10 mouse knee joints with a collagenase solution led to clear and progressive osteoarthritic lesions in the injected joint within two weeks. The incidence of osteoarthritis was five out of five mice after the injection of collagenase. The injection of the contralateral knee joint with physiological saline had no effects on the joint structures. The osteoarthritic alterations were characterized by

fibrillation of cartilage and matrix depletion in the femorotibial joint in the early stages (7 days) and erosion of cartilage and subchondral bone and osteophyte formation in the later stages (21 days en 42 days). Also proliferative responses could be observed in the collateral ligaments and menisci from day 7 and later.

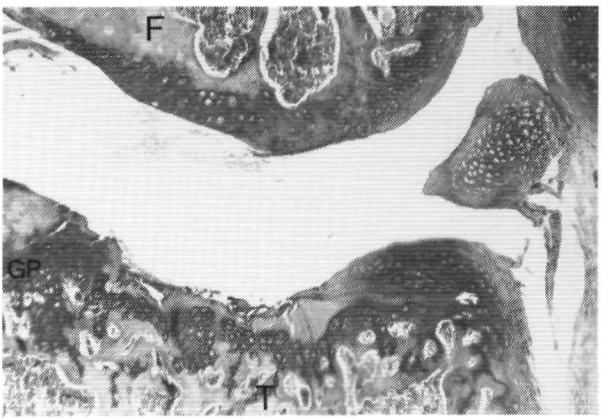

Figure 6: Medial side of the femorotibial joint of a right knee 42 days after injection of 1% collagenase. Erosion of cartilage on the tibial plateau and femoral condyle and of subchondral bone on the tibial plateau is clear. Sclerosis of subchondral bone below the cartilage lesions can be seen (original magnification X 40). See also legend figure 1.

Collagenase is an enzyme that will damage mainly the joint structures which contain collagenase type I in the extracellular matrix, such as tendons, ligaments and menisci. Although collagenase is frequently used in the digestion of cartilage slices (Glowack et al. (1983)), intact articular cartilage was resistent to the proteolytic activity of the collagenase we used in our experiments. In vitro incubation of anatomically intact patellae (undamaged cartilage) in 1% collagenase did not result in breakdown of the articular cartilage (data not shown). However, one day after collagenase injection in the knee joints a moderate loss of safranin O staining in the femoropatellar cartilage could be seen which was already overcome after 3 days. This depletion is to our opinion not due to the collagenase itself but to events in the joint tissues induced with collagenase e.g. enzyme release

from synoviocytes or Il-1 generation. The loss of safranin O staining after 1 day (femoropatellar joint) did not seem to be associated with the localization of the osteoarthritic lesions at later stadia (femorotibial joint).

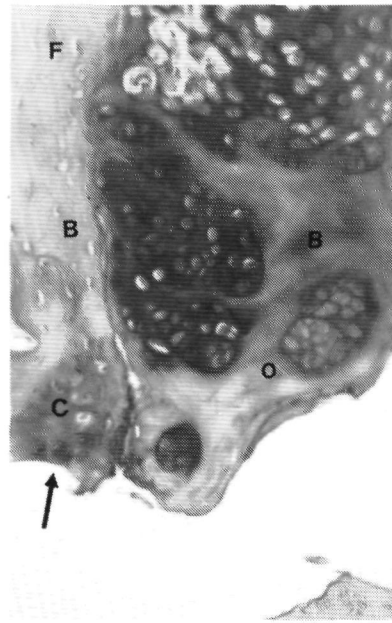

Figure 7: Detail of a femoral osteophyte in the right knee joint, 42 days after injection of 1% collagenase. A small chondrocyte cluster can be seen in the remaining articular cartilage (arrow) adjacent to the osteophyte (original magnification X 250). See also legend figure 1.

A major effect of the collagenase injection is the medial dislocation of the patella, a trait which is also associated with spontaneous osteoarthritis observed in STR/IN and STR/ORT mice (Schünke et al. (1988), Walton (1977 a,b and c, 1979)). According to Walton, the patellar dislocation may be an important factor in the aetiology of the osteoarthritis while Schünke et al consider it to be the consequence of joint instability and deformity as a result of the osteoarthritic process (Schünke et al. (1988), Walton (1977a,b and c, 1979)). Collagenase injection resulted already after one day in a high incidence of patellar dislocation but osteoarthritic lesions were only observed at a later stage. We

think that the patellar dislocation indicates the presence of joint instability either as an outcome of collagenase injection or as a result of intrinsic changes in the joint leading to this instability in the case of the spontaneous models. The joint instability eventually results in development of osteoarthritic changes in the joint. The joint instability might lead to metabolic alterations in certain chondrocytes resulting in osteoarthritis in the area of the affected chondrocytes. Altman observed, in his study with STR/ORT mice, chondrocyte inactivation in cartilage areas prone to osteoarthritis before the histological evidence of degeneration (Altman (1981)). The altered chondrocyte metabolism might be a result of mechanical stress in the osteoarthritis prone cartilage.

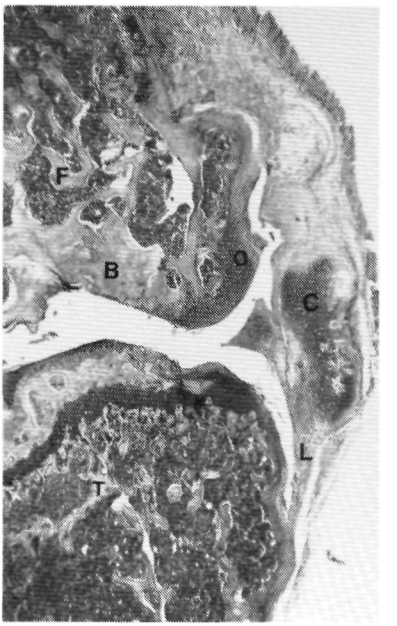

Figure 8: Medial side of a femorotibial joint of a right knee 42 days after injection of 1% collagenase. Erosion of cartilage, sclerosis of subchondral bone and pronounced cartilaginous changes in the collateral ligaments are present (original magnification X 40). See also legend figure 1.

The most severe osteoarthritic changes we observed in the

collagenase-induced model were present on the medial side of the femoropatellar joint. This is also the case in the spontaneous models in the STR/IN and STR/ORT mice were the osteoarthritic changes are almost totally confined to medial side of the femorotibial joint (schünke et al. (1988), Walton (1977 a,b and c, 1979)). However, osteoarthritic lesions in the knee joint of old (18 month) C57Bl mice are not restricted to the medial side of the femoropatellar joint but can also be seen on the lateral side (figure 9). This indicates that the etiopathogenesis of osteoarthritis in mice is multifactorial and can lead to a diversity of joint alterations.

The formation of cartilage clefts and eburnation of the non-calcified cartilage, extending to the tidemark, was followed by the loss of calcified cartilage exposing the subchondral bone to the surface in both the collagenase-induced and spontaneous osteoarthritis. Under the cartilage erosions the subchondral bone had become increasingly thicker. Similar phenomena are described for the STR/IN and STR/ORT strains by Walton and Schünke et al (Schünke et al. (1988), Walton (1977 a,b and c, 1979)) and were observed in the spontaneous osteoarthritis in the knee joint cartilage of old C57Bl10 mice at our laboratory (figure 9).

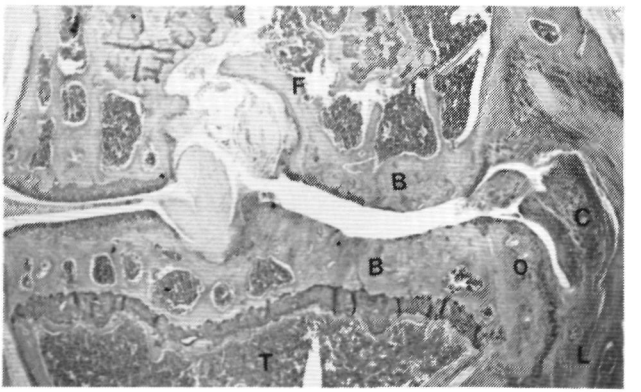

Figure 9. Spontaneous osteoarthritis in the knee joint of a 18 month old C57Bl10 mouse. Erosion of cartilage and subchondral bone is evident. Sclerosis of bone can be seen below the eroded sides and osteophytes are present. (original magnification X 40)

In both the induced and the spontaneous osteoarthritis, the cartilage adjacent to the observed cartilage clefts and erosions was more acellular than the cartilage in control joints. The cells remaining in the proteoglycan depleted matrix, as indicated by a loss of safranin O staining, were most times surrounded with a pericellular halo of strongly staining matrix. Chondrocyte clusters were present but only in small numbers. Similar observations were done in the spontaneous osteoarthritis in other mouse strains (Schünke et al. (1988), Walton et al. (1977 a,b and c, 1979)). In preliminary studies we observed that the glycosaminoglycan synthesis, measured by the incorporation of radiolabeled sulfate, was elevated in the osteoarthritic cartilage in the collagenase-induced model (data not shown). The presence of the strong pericellular staining and the elevated glycosaminoglycan synthesis indicate that the chondrocytes present in the relatively acellular matrix have a high proteoglycan synthesis rate. As the surrounding matrix had a reduced safranin O staining the synthesized proteoglycans do not remain in the osteoarthritic cartilage.

The osteoarthritic alterations in the mouse knee joints are not confined to the articular cartilage and bone but also other joint structures are involved in the degenerative process. Metaplastic changes in the synovial tissue and collateral ligaments are frequently observed in the spontaneous models of murine osteoarthritis (Sokoloff (1956), Sokoloff & Jay (1956), Walton (1977 a,b and c, 1979)). Also cartilaginous changes in the collateral ligaments and ossification processes in the menisci are common in osteoarthritic mouse knee joints (Sokoloff (1956), Sokoloff & Jay (1956), Walton (1977 a,b and c, 1979). Similar alterations were seen in the collagenase-induced osteoarthritis here described.

A moderate inflammation of the joint could be seen one day after the injection of collagenase. Cellular infiltration in the synovium and some exudation in the joint cavity were present. The exudate was transient and had already nearly completely disappeared after three days while a minor infiltration of the synovium with inflammatory cells was present throughout the entire study. When compared with the synovitis in the murine arthritis models we routinely use in our laboratory, the inflammation in the collagenase osteoarthritis model was, with respect to inflammatory

cell influx, moderate and a similar degree of inflammatory cell influx in our arthritis models does not result in loss of Safranin O staining and cartilage damage in these models (Kruysen et al. (1983), Kruysen et al. (1985), Van den Berg et al. (1981)). So, the role of the inflammatory cells in the pathogenesis of the collagenase-induced osteoarthritis will be a minor one.

The joint alterations in the collagenase-induced osteoarthritis model were similar to the alterations observed in spontaneous osteoarthritis in aged mice. These changes closely resemble the corresponding lesions of articular cartilage in man, making the mouse a suitable experimental model for osteoarthritis studies (Silberberg & Silberberg (1971)). The collagenase-induced osteoarthritis model has several advantages compared to the currently used experimental models. The use of mice makes it possible to study osteoarthritic phenomena in whole knee joint sections of large animal groups in highly inbred strains. The intra-articular injection of collagenase in young animals (10 weeks) results in joint alterations similar to those observed in spontaneous osteoarthritis in old mice but within a limited time span (a few weeks). In conclusion, the use of young mice and the non-time consuming setup of the experiments makes it possible to study experimental osteoarthritis in a convenient way at the lowest costs.

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CHAPTER 8

DEVELOPMENT OF OSTEOARTHRITIS MODELS IN MICE BY "METABOLICAL" AND "MECHANICAL" ALTERATIONS IN THE KNEE JOINTS

Peter M. van der Kraan, Elly L. Vitters, Levinus B.A. van de Putte and Wim B. Van den Berg

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ABSTRACT

Male , 10 week old, C57Bl10 mice received a single intra-articular injection in the knee joints with papain, iodoacetate or collagenase. This led to osteoarthritic lesions, such as matrix depletion, chondrocyte proliferation and osteophyte formation, in the injected knee joints within several weeks. After injection of iodoacetate and papain the main osteoarthritic alterations were localized in the femoropatellar joint while injection of collagenase led to marked osteoarthritic lesions in the femorotibial joint. The mechanism of induction of these alterations appears to differ for iodoacetate and papain on one site and collagenase on the other site. Data are presented that collagenase injection results by way of damaging ligaments and tendons, to destabilization of the knee joint eventually leading to osteoarthritic alterations. In contrast, injection of papain or iodoacetate directly interferes with cartilage metabolism resulting in this way to osteoarthritic changes.

INTRODUCTION

Osteoarthritis is an age-related disease of diarthrodial joints in which the predominant characteristic is degeneration of articular cartilage[1]. This process is often associated with proliferation of chondrocytes and formation of new cartilage and bone at joint margins leading to the formation of osteophytes. Osteoarthritis is presently considered as the clinicopathological consequence of various etiologic factors and most researchers agree that osteoarthritis is not a single disease entity[1,2]. The various forms of human osteoarthritis can be characterized as being the ultimate result of abnormal biomechanical stress or intrinsic or acquired abnormalities of articular cartilage. The final common pathway of this multifactorial disease is disturbed cartilage integrity.

The changes occurring in the articular cartilage, as a result of the osteoarthritic process, are manyfold. Hydration of the extracellular matrix and the extractability of proteoglycans is increased in osteoarthritic cartilage compared to normal cartilage, both in human and experimental osteoarthritis[3-5]. Synthesis of cartilage proteoglycans is increased while the quantity of proteoglycans is reduced in osteoarthritic cartilage, indicating a highly increased breakdown of proteoglycans[6-9]. Also changes in the biochemical properties of proteoglycans from osteoarthritic cartilage are reported[5,11-15]. The normally non-proliferating articular cartilage chondrocytes are stimulated to divide as a result of the osteoarthritic process[3,16-24].

Besides changes in the articular cartilage, also other joint structures undergo alterations in the osteoarthritic joints. Formation of osteophytes at the joint margins is highly associated with the osteoarthritic changes in the articular cartilage[16-26]. Proliferation of synovial cells is observed both in human osteoarthritis and in animal models[18,21,24,27-31]. Another phenomenon seen in osteoarthritic joints is sclerosis of subchondral bone below the degenerated cartilage[17,24,25,32,33]. These observations point to anabolic processes in the joint in addition to the catabolic degeneration of articular cartilage.

Since the pathogenesis of osteoarthritis can not adequately be studied in humans, interest has been directed to animal models

of osteoarthritis which simulate the human disease. Spontaneous osteoarthritis has been studied in mice, guinea pigs and dogs[4,24,25,33-40]. In addition, osteoarthritis has been induced in animal knee joints in two different ways, induction of instability of the joint by surgical methods and intra-articular injection of chemicals interfering with cartilage metabolism. Induction of joint instability has been carried out by (partial) meniscectomy, frequently in combination with dissection of collateral ligaments, and dissection of cruciate ligaments or myectomy[5,9,14,18-23,27,41-44]. The intra-articular injection of papain or sodium iodoacetate leads to osteoarthritic alterations in the injected joints[45-57].
Since osteoarthritis is considered to be the final result of a multifactorial pathogenesis we have used three different ways of osteoarthritis induction in C57Bl10 mice. Knee joints of the mice were injected with papain, sodium iodoacetate or collagenase. Papain induces degeneration of cartilage proteoglycans, sodium iodoacetate is an inhibiter of cell metabolism and collagenase mainly attacks joint structures containing collagen type I, such as tendons and ligaments.

MATERIALS AND METHODS

ANIMALS

Male C57Bl/10 mice at 10 weeks of age were used in all experiments. The animals were kept in boxes in an air conditioned room at constant temperature and were fed a standard laboratory diet (Hope Farms, Deventer, The Netherlands) with access to tap water ad libitum.

INDUCTION OF OSTEOARTHRITIS

The right knee joints of the mice were injected once, intra-articularly through the patellar ligament, with a 6 microliter solution of papain, sodium iodoacetate or collagenase. The left control knee joint was injected with 6 microliter physiological saline. Papain (Type IV, double crystallized, 15 units/mg, Sigma, St. Louis, MO) was used in a concentration of 0.5%, 1.0% and 2.0% (w/v) and these solutions were supplemented with 0.03 M L-cysteine.HCl (Sigma) to activate the papain. Sodium iodoacetate (Sigma) was injected in a

concentration of 0.1%, 0.5% and 1.0% (w/v) while collagenase (Clostridial, 248 units/mg, Worthington Biochemical Corporation, Freehold, New Jersey) was injected in the following concentrations 0.5%, 1.0% and 1.5% (w/v). Before injection the solutions were filtered through a 0.2 μm filter to remove bacteria.

HISTOLOGY

Groups of 5 mice injected with papain or collagenase were killed by cervical dislocation 1, 3, 7, 21 and 42 days after intra-articular injection while a group of mice injected with iodoacetate was killed also after 64 days. Carefully dissected knee joints were fixed in phosphate-buffered formalin (pH 7.4) for 5 days and subsequently decalcified in 5% formic acid for four days. Standard processing of the knee joints in a automatic tissue processing apparatus was followed by embedding of the knee joints in paraffin wax. Frontal whole knee joint sections were prepared (6 μm) and stained with safranin O and fast green.

DETERMINATION OF JOINT SWELLING

Soft tissue joint swelling after intra-articular injection was assayed on day 1, 3, 7, 21, 41 and 64 (iodoacetate) by the ^{99}mTc-pertechnetate uptake method as described previously[58]. In short, mice were injected with 15 μCi ^{99m}Tc and sedated with choral hydrate (intraperitoneally). After 30 min the quantity of radiolabel in the right and the left knee joints was determined by measuring the gamma radiation with a collimated NaI-scintillation crystal while the knee joint was in a fixed position. Joint swelling was scored as the ratio of the radiolabel in the right and the left knee joint.

GLYCOSAMINOGLYCAN SYNTHESIS

After 1, 3, 7, 21, 42 and 64 (iodoacetate) days groups of 5 mice were killed and the whole patellae along with a standard amount of surrounding tissue, were dissected from both the left and the right knee joints, according to the method of van den Berg et al.[59]. Patellae were incubated in RPMI 1640 DM medium (Flow Laboratories, Irvine, UK) containing 20 μCi [^{35}S]sulfate (1200 Ci/mmol, Radiochemical Centre Amersham,

Amersham, UK) for 2 hours at 37 °C in a humidified 5% CO_2 atmosphere. The inorganic sulfate concentration was 0.8 mM. After incubation the patellae were washed twice with physiological saline to remove non-incorporated label and fixed in 4% phosphate-buffered formalin (pH 7.4). The patellae were isolated from the surrounding tissue after overnight decalcification in 5% formic acid. The patellae were digested with Lumasolve (Hicol, Oud-Beijerland, The Netherlands) and the incorporated radiolabel was determined by liquid scintillation counting. Radiolabel is incorporated almost exclusively in the proteoglycans of the patellar cartilage. Hardly any label is found in the underlying bone and bone marrow cells[60].

STATISTICAL EVALUATION

The glycosaminoglycan synthesis was evaluated by means of analysis of variance in combination with the two tailed Student T-test. Joint swelling was evaluated with the Wilcoxon Rank Sum test. A P value <0.05 was considered to be significant.

RESULTS

EFFECT OF INTRA-ARTICULAR INJECTION ON JOINT SWELLING

Papain was injected intra-articularly in the right knee joint of mice in three different concentrations, 0.5%, 1.0% and 2.0%. Only 1 day after the injection of 2% papain the right knee joint was significantly thicker than the left knee joint injected with physiological saline, as determined by the amount of radiolabel in the knee joints (figure 1A). At later stages, a significant joint swelling was not observed at any concentration. The injection of 0.5% and 1.0% iodoacetate resulted in a significant joint swelling 1 day after injection while the injection of 0.1% did not result in a significant response (figure 1B). The injection of collagenase had the most pronounced effects on joint swelling. All concentrations (0.5%, 1.0%, 1.5%) induced a significant joint swelling on day 1 and day 3 after intra-articular injection (figure 1C).

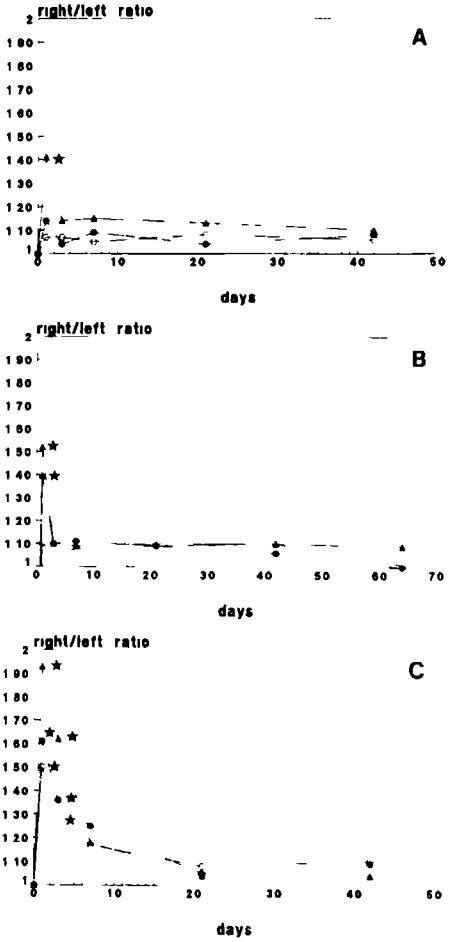

Figure 1: Joint swelling after injection with papain, collagenase or iodoacetate. Mice were injected with radioactive technetium and after 30 min. the quantity of radiolabel in the left and the right knee joint were determined. Joint swelling was scored as the ratio of the radiolabel in the right and the left knee joint. Expressed are mean values ± s.d. of at least five mice (A papain ○ 0.5%, ● 1.0%, ▲ 2.0%, B iodoacetate ○ 0.1%, ● 0.5%, ▲ 1.0%, C collagenase ○ 0.5%, ● 1.0%, ▲ 1.5%, ★ = significant different from control joints)

EFFECT OF INTRA-ARTICULAR INJECTION ON GLYCOSAMINOGLYCAN SYNTHESIS

The intra-articular injection of papain resulted in an concentration dependent inhibition of glycosaminoglycan synthesis in patellar cartilage after 1 day (figure 2A). After 3 days, the glycosaminoglycan synthesis in the patellae from mouse knees injected with 0.5% or 1.0% papain was significantly elevated while the synthesis in the patellae from knee joints injected with 2% papain was still inhibited. Seven days after the injection of papain, there was a slight but non-significant elevation of glycosaminoglycan synthesis in the patellae of all papain injected knee joints.
Injection of iodoacetate in the knee joints resulted in a inhibition of patellar glycosaminoglycan synthesis up to more than 80% after 1 day (figure 2B). The glycosaminoglycan synthesis in the patellae of knee joints injected with 1% iodoacetate continued to be inhibited from day 1 till day 64. However, already 3 days after injection of 0.1% iodoacetate the glycosaminoglycan synthesis in the patellae was significantly increased compared to the control patellae. The patellae from the knee joints injected with 0.5% iodoacetate showed a normal overall synthesis of glycosaminoglycans in the whole patellae after 3 and 7 days but was significantly decreased at later stadia.
Figure 2C shows the effect of collagenase injection on the patellar glycosaminoglycan synthesis. After one day, injection of 1.0% or 1.5% collagenase resulted in a significant inhibition of patellar glycosaminoglycan synthesis while a significantly elevated synthesis was observed after injection of 0.5% or 1.5% on day 7. An increased synthesis was also observed at all concentrations on day 21 and at 1.0% and 1.5% on day 42 but this was only significant for 1.0%.

HISTOLOGICAL OBSERVATIONS

Three concentrations of papain, iodoacetate or collagenase were used to induce osteoarthritic changes in the right knee joints of C57Bl10 mice. The joint alterations were essentially similar at all three concentrations and only quantitative

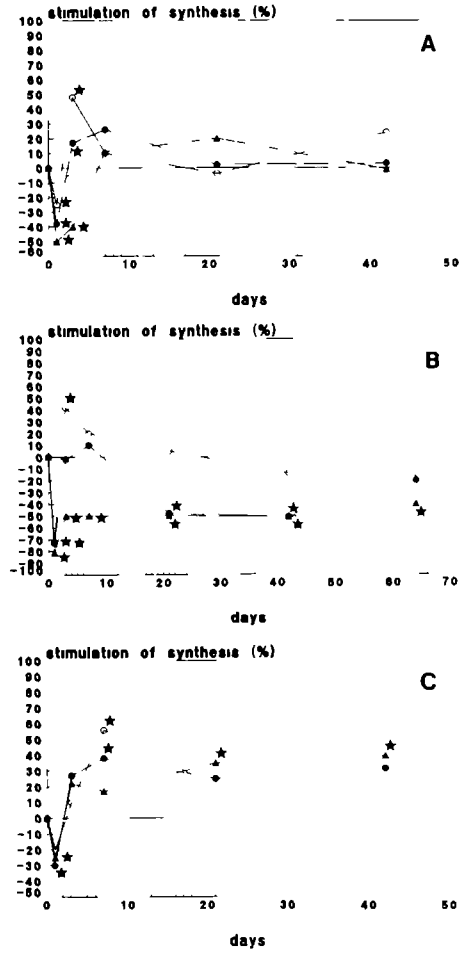

Figure 2: Glycosaminoglycan synthesis, as measured by sulfate incorporation, in patellar cartilage after injection of papain, iodoacetate or collagenase. The glycosaminoglycan synthesis is compared to the glycosaminoglycan synthesis in the left control joint. Expressed are the mean values ± s.d. of at least five patellae (A papain ○ 0.5%, ● 1.0%, ▲ 2.0%, B iodoacetate ○ 0.1%, ● 0.5%, ▲ 1.0%, C collagenase ○ 0.5%, ● 1.0%, ▲ 1.5%, ★ = significant different from control joints).

differences were observed. The results of 1% papain, 1% collagenase and 0.5% iodoacetate will be reported and only major differences at other concentrations will be discussed. The results are summarized in table 1 and table 2. Table 1 showes the changes in the articular cartilage while table 2 presents the changes in other joint structures and the grade of inflammation. None of the control knee joints showed any evidence of osteoarthritic alterations.

DAY 1

One day after the injection of papain there was a pronounced depletion of safranin O staining in all the non-calcified articular cartilage of the joint, indicating a reduced proteoglycan concentration (figure 3). Injection with 2% papain even led to disruption of non-calcified cartilage from the calcified cartilage along the tidemark or to degradation of the cartilage surface. The effect of papain on articular cartilage was most extreme on the central part of the patella. The injection of iodoacetate had no effect on the safranin O staining or structure of the articular cartilage. One day after the intra-articular injection of collagenase a medial translocation of the patella was observed in 4 out of 5 injected joints (figure 4). The cartilage of the patellaris femoris and the patella showed a mild loss of safranin O staining while the cartilage of the femorotibial joint was normally stained.

Signs of inflammation were observed in all three models. A low grade synovial leukocyte infiltration and exudation of inflammatory cells in the joint cavity was found. This was most pronounced in the cavity of the femoropatellar joint.

DAY 3

The depletion of safranin O staining in the femorotibial joint was less marked three days after papain injection than one day after papain injection. This was not observed in the femoropatellar joint. The intermediate zone of the femorotibial cartilage showed signs of chondrocyte proliferation and cell clusters could be seen in the safranin O-depleted cartilage. Acellular areas in the tibial cartilage could be seen in the group injected with 2% papain. The knee

Table 1: Histological observation of alterations in the articular cartilage of papain (1%), iodoacetate (0.5%) and collagenase (1%) injected knee joints of male C57Bl10 mice (0= no alterations, +++= pronounced pathological alterations, P=papain, I=iodoacetate, C=collagenase, ND= not determined).

DAY		1	3	7	21	42	64
Cartilage	P	++	++	++	++	++	ND
fibrilation	I	0	0	0	+	+	++
	C	0	0	0	++	++	ND
Erosion of	P	+	+	+	++	++	ND
non-calcified	I	0	0	0	0	+	++
cartilage	C	0	0	0	++	+++	ND
Erosion of	P	0	0	0	0	0	ND
calcified	I	0	0	0	0	0	0
cartilage	C	0	0	0	+	+++	ND
Chondrocyte	P	+	+	+	++	++	ND
death	I	+	++	++	++	++	++
	C	0	0	0	++	+++	ND
Chondrocyte	P	0	+	++	++	++	ND
clusters	I	0	0	0	+	+	+
	C	0	0	0	+	+	ND
Loss of	P	+++	++	+	++	++	ND
safranin O	I	0	0	+	++	+++	+++
staining	C	+	0	+	++	+++	ND

joints of mice injected with iodoacetate showed a very mild depletion of staining in the central part of the femoropatellar joint but other changes were not observed. Medial dislocation of the patella was evident in four of five mice injected with collagenase. The depletion of safranin O staining in the femoropatellar joint was decreased compared to the depletion observed one day after collagenase injection.

Table 2: Histological observations of inflammation and alterations in non-articular cartilage joint structures in murine C57Bl knee joints after injection with papain (1%), iodoacetate (0.5%) and collagenase (1%) (0= no alterations, +++= pronounced pathological alterations, P=papain, I=iodoacetate, C=collagenase, ND= not determined).

DAY		1	3	7	21	42	64
Patella	P	0	0	0	0	0	ND
displacement	I	0	0	0	0	0	0
	C	++	++	++	++	++	ND
Erosion of	P	0	0	0	0	0	ND
bone	I	0	0	0	0	0	0
	C	0	0	0	+	+++	ND
Sclerosis of	P	0	0	0	0	0	ND
subchondral	I	0	0	0	0	0	0
bone	C	0	0	0	+	++	ND
Osteophyte	P	0	0	0	+	++	ND
formation	I	0	0	+	++	++	++
	C	0	0	0	++	+++	ND
Synovium	P	0	++	+	0	0	ND
hyperplasia	I	0	++	++	+	+	0
	C	0	0	++	+++	+++	ND
Inflammatory	P	++	+	0	0	0	ND
exudation	I	++	+	0	0	0	0
	C	++	+	0	0	0	ND
Synovial	P	++	+	+	+	0	ND
infiltration	I	++	+	+	+	0	0
	C	+	+	+	+	+	ND

The exudate of inflammatory cells in the joint cavity was greatly reduced compared with the exudate present after one day, in all three models studied. Only a few cells could still

be seen in the joint cavity. A mild infiltration of the synovium with inflammatory cells could still be observed.

DAY 7

Seven days after papain injection the non-calcified patellar cartilage showed areas devoid of cells lying next to hypercellular areas with numerous cell clusters (figure 5). Highly safranin O-staining halos were seen around the cell clusters. Proliferation of cells and cluster formation was also evident in the femoropatellar cartilage and the menisci. The synovium appeared to be slightly thickened in the papain injected knee joints.

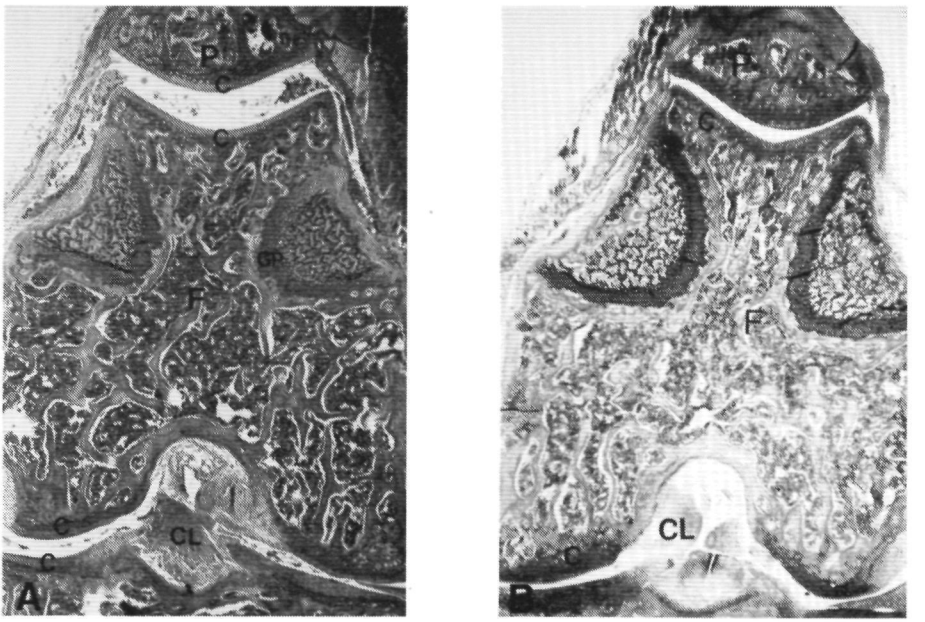

Figure 3: C57Bl10 knee joint one day after the intra-articular injection of 1% papain. Note the depletion of safranin O staining in the non-calcified cartilage (frontal section, original magnification 40x, safranin O-fast green,
A - injected joint, B - control joint). P = patella,
F = femur, T = tibia, GP = growth plate, C = cartilage,
B = bone, CL = cruciate ligaments, O = osteophyte,
L = ligament.

Cell death was observed in the central part of the patella and to a minor extend in the patellaris femoris in the knee joints of mice injected with iodoacetate (figure 6). The same cartilage areas also showed a moderate depletion of safranin O staining. In the depleted matrix some cells were present which markedly stained with safranin O. Proliferation of chondrocytes was not observed. The femorotibial joint also showed a slight depletion of safranin O staining after injection with 1% iodoacetate. Early signs of osteochondrophyte formation was evident at the margins of the femoropatellar joint and in a few animals, to a minor extent, also at the margins of the femorotibial joint.

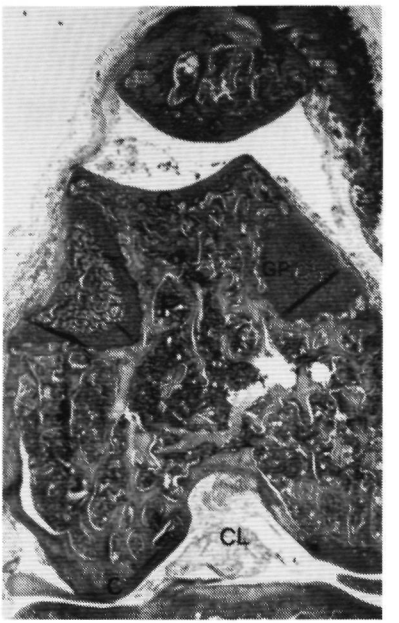

Figure 4: Patellar displacement to the medial side of the joint one day after injection of 1% collagenase (frontal section, original magnification 40x, safranin O-fast green, see for legend and control joint figure 3).

In the collagenase-injected joints the cartilage of the femorotibial joint showed a loss of safranin O staining while the staining of the femoropatellar cartilage was comparable

with the staining of control joints. The synovial tissue on the medial side of the was joint markedly thickened by proliferation of synovial cells while the synovium on the lateral side was only slightly thickened (figure 7). Inflammatory cell infiltration in the synovium tissue was not observed.

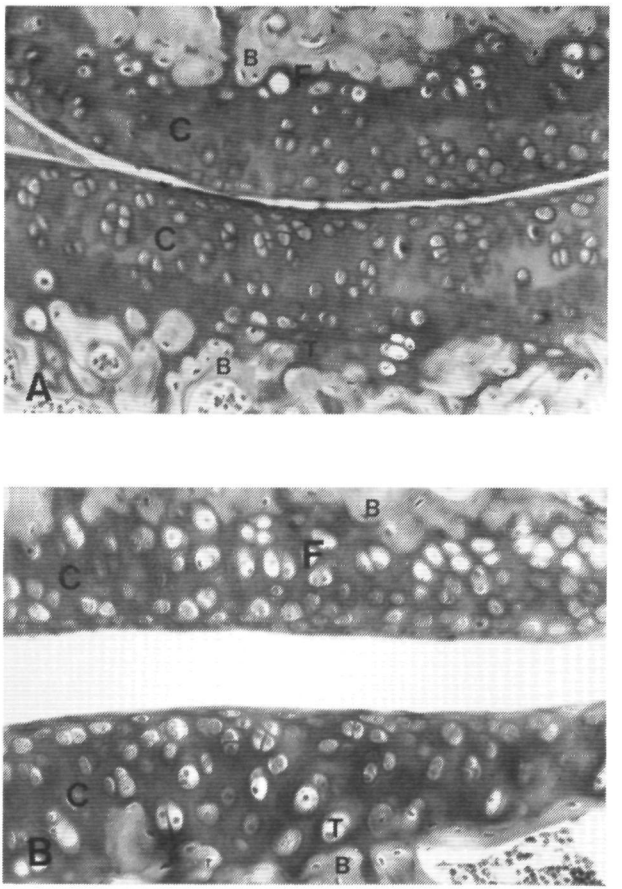

Figure 5: Proliferation of chondrocytes in the femoropatellar joint after the intra-articular injection of papain (day 7, frontal section, original magnification 250x, safranin O, fast green, see for legend figure 3, A - injected joint,
B - control joint)

DAY 21

Injection with papain led to acellular areas in the patellar cartilage lacking significant safranin O staining while other areas showed hypercellularity and a strongly staining matrix indicating cartilage repair (figure 8). Loss of cartilage in the depleted areas was seen in 2 out of 5 animals. Formation of osteophytes could be seen most pronounced at the medial side of the femoropatellar joint but in 1 out of 5 animals also at the medial side of the femorotibial joint.

In the knees injected with iodoacetate a pronounced depletion of the central part of the patella was observed while some areas adjacent to the central part showed chondrocyte clusters with a markedly stained pericellular matrix. Also the femorotibial areas showed signs of chondrocyte proliferation. Osteophyte formation was seen at the margins of the femoropatellar joint.

Figure 6: Femoropatellar knee joint 7 days after the injection of 0.5% iodoacetate. Formation of osteophytes at the margins of the joint and acellular areas in the central part of the patella can be seen (frontal section, original magnification 100x, safranin O-fast green, see for legend figure 3)

A loss of safranin O staining was observed in the cartilage of the femorotibial joint of the collagenase-injected knees. This was most pronounced at the medial side of this joint. The depleted matrix appeared to be hypocellular but the cells

still present in the depleted matrix demonstrated a heavily stained pericellular matrix.

DAY 42

The knee joints injected with papain showed in the patellar cartilage the presence of chondrocyte clusters in an intensely stained extracellular matrix adjacent to acellular safranin O-depleted areas. Pieces of cartilage were disrupted from the degenerated cartilage. This was to a lesser extent also observed in the femoris patellaris. Osteophytes were observed on the margins of the femoropatellar joint, mainly on the medial side of this joint (figure 9). Osteophytes on the femorotibial joint were less common. There were no signs of inflammation in the joints.

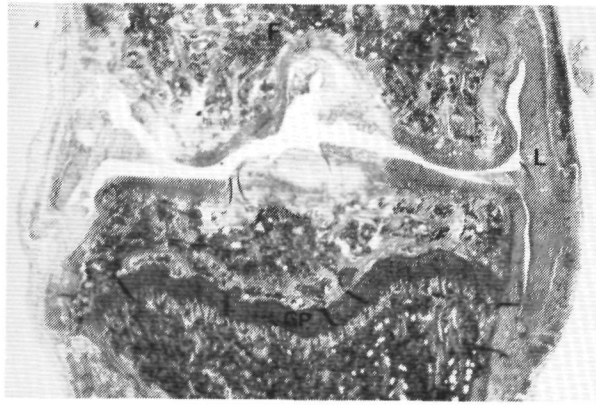

Figure 7: Proliferation of synovial cells seven days after the injection of 1% collagenase, note the difference in proliferation on the medial side of the joint in contrast to the proliferation on the lateral side of the joint (frontal section, original magnification, safranin O-fast green, 40 x, see for legend figure 3).

Injection of iodoacetate led to the formation of large osteophytes on the margins of the femoropatellar joint (figure 10) and sometimes to small osteophytes on the margins of the femorotibial joint. The osteophyte formation was most marked at the medial side of the joints. The central part of the

patella and to a lesser extent the opposite femoral cartilage showed a significant loss of safranin O staining. Areas neighboring depleted areas were hypercellular in some of the animals. Loss of safranin O staining was also observed in the cartilage of the tibial plateau. Signs of inflammation were absent in the joints.

The injection of collagenase resulted in a gross change in the appearance of the knee joints. Due to osteophyte formation, thickening of the collateral ligaments and synovium and erosion of cartilage and bone the height of the joints was decreased and the width was increased. The osteoarthritic lesions were severe. Cartilage at the medial side of the femorotibial joint was totally eroded and the subchondral bone had become sclerotic (figure 11). In two out of five mice the subchondral bone on the medial side of the femorotibial joint was exposed to the surface. Changes on the lateral side of the joint were always less pronounced than on the medial side of the joint. Formation of cartilage-like tissue, as indicated by safranin O staining, was observed in the collateral ligaments.

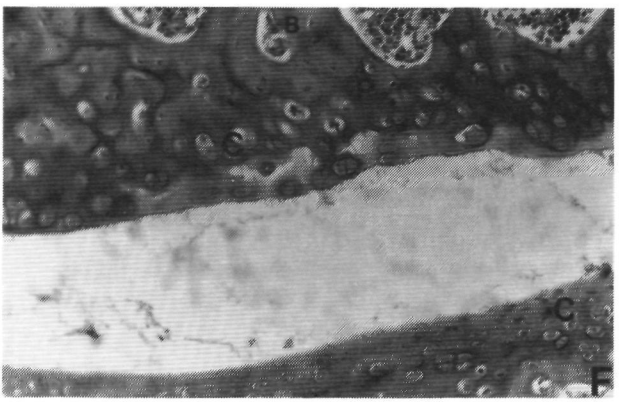

Figure 8: Cartilage of the femoropatellar joint 21 days after the injection of 1% papain. Acellular areas in the patellar cartilage with little safranin O staining are observed lying next to highly stained cellular areas (frontal section, original magnification 250x, safrainin O-fast green, see for legend figure 3).

DAY 64

Sixtyfour days after the injection of iodoacetate the cartilage in the central parts of the patella appeared to be thinner in the iodoacetate injected knee joints than in the physiological saline injected knee joints. The central part of the patella was depleted of safranin O staining while chondrocyte clusters could be seen in non-depleted parts. Osteophytes were present most marked at the medial side of the femoropatellar joint but also at the other joint margins.

DISCUSSION

A single injection of male C57Bl10 mice with papain, iodoacetate or collagenase leads to osteoarthritic-like lesions in the injected knee joints after a few weeks. Interference with joint stability by injection of collagenase in the knee joints results in joint changes similar with changes observed in murine osteoarthritis (compare fig. 11 and fig. 12). Injection of papain or iodoacetate leads to degenerative alterations in the knee joints, as a result of cartilage damage, with many aspects in common with osteoarthritis.

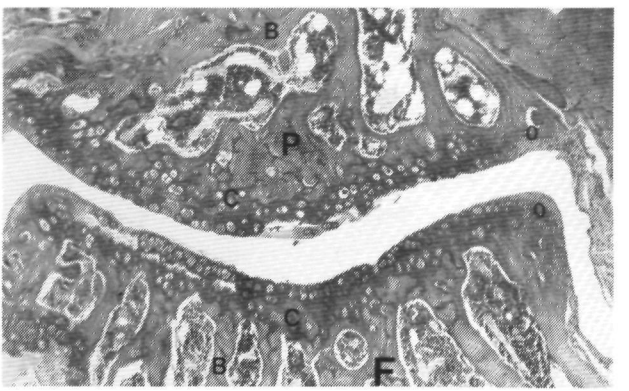

Figure 9: Femoropatellar joint 42 days after the injection of 1% papain. Cartilage fragments are disrupted from the cartilage surface and osteophytes are present on the margins of the joint (frontal section, original magnification 100x, safranin O-fast green, see for legend figure 3).

The injection of papain, iodoacetate or collagenase leads in the initial phase to a moderate inflammatory reaction in the knee joint. This inflammatory response was characterized by synovial infiltration with inflammatory cells and the presence of these cells in the joint cavities. The exudate was transient in all three models and had almost disappeared three days after injection. A low grade synovial infiltrate could be observed until day 21. An inflammatory response was also indicated by the joint swelling observed after injection of papain, iodoacetate or collagenase. The joint swelling, as indicated by radiolabel uptake of the knee joints, was also transient and significant joint swelling was not observed by three days after intra-articular injection. The joint swelling induced by the injection of collagenase was higher than could be expected by the histological evaluation of the inflammatory response in the injected knee joints[58]. The collagenase injection probably has a direct effect on endothelial structures in the joint and leads by this way to a more pronounced joint swelling than expected by the observed inflammation.

The inflammatory response in all three models was low grade when compared to the inflammatory response in the murine arthritis models we routinely use in our laboratory[61-63]. A grade of inflammation in the arthritis models comparable with the grade observed in the osteoarthritis models does not result in a loss of safranin O staining or cartilage damage in the later stages in the arthritis models. Therefore, the role of the inflammatory response in the induction of the osteoarthritic lesions will be an insignificant one. In addition, 18 month old C57Bl10 mice with spontaneous osteoarthritis also showed a low grade synovial infiltrate. An inflammatory reaction is also observed in other animal models of osteoarthritis and is regularly a component of human osteoarthritis[64-69].

Injection of collagenase resulted already after one day in a high incidence of patellar dislocation to the medial side of the injected knee joint. Injection of papain, iodoacetate or physiological saline never led to patellar displacement. Patellar dislocation to the medial side of the knee joints is also associated with spontaneous osteoarthritis in STR/IN and

STR/ORT mice[25,33,37-39]. The patella dislocation demonstrates the presence of joint instability either as a result of inherent joint malformation (STR/IN and STR/ORT strains) or as the consequence of the collagenase injection. The effect of collagenase on collagen type I containing joint structures, such as ligaments and tendons, induces the observed joint instability. [c]ollagenase (1%) had no degradative effect on the anatomically intact cartilage of patellae after in vitro incubation but degrades sliced bovine cartilage under the same conditions (data not shown).

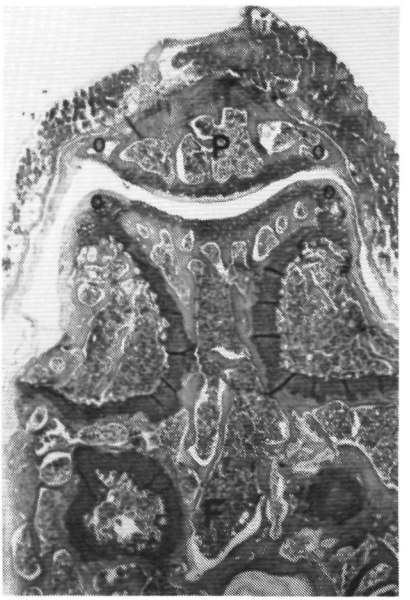

Figure 10: Formation of osteophytes on the medial side of the femoropatellar joint 42 days after the injection of 0.5% iodoacetate. The cartilage of the femoropatellar joint clearly shows depletion of safranin O staining (frontal section, original magnification 40X, safranin O-fast green, see for legend figure 3).

The injection of papain and iodoacetate had the most pronounced effects on the cartilage of the femoropatellar joint while the injection of collagenase resulted in marked pathological changes in the femorotibial joint. The central

parts of the patellae, and to a lower extent the opposite side on the femur, expressed the most distinct depletion of safranin O and cell death after the injection of papain or iodoacetate. A similar predisposition was observed in the antigen (methylated bovine serum albumin) induced arthritis model in C57Bl10 mice in our laboratory[59]. Moreover, in vitro incubation of murine patellae with hydrogen peroxide resulted in more severe inhibition of proteoglycan synthesis and higher cell death in the central chondrocytes than in more peripheral situated chondrocytes[70]. This indicates that chondrocytes from certain areas of knee joint joint cartilage are more vulnerable to the effects of harmful compounds than chondrocytes from other areas. As the central part of the patella in mice is one of the load bearing parts of the joint, the susceptibility of chondrocytes in this area might be related to the in vivo stress on these chondrocytes.

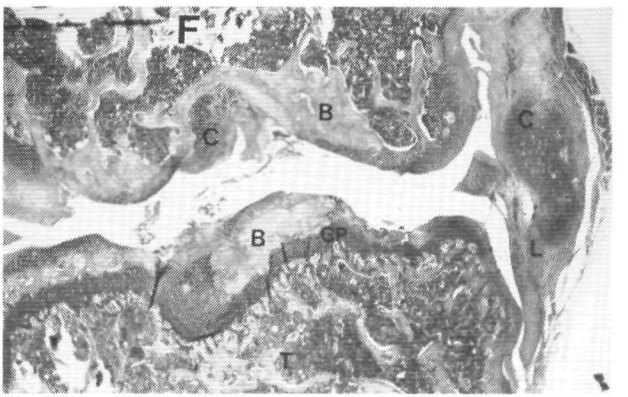

Figure 11: Destruction of the femorotibial joint 42 days after the injection of 1% collagenase. Erosion of bone is clear on the medial side of the tibial plateau and the ligaments show cartilaginous alterations (frontal section, original magnification 40x, safranin O-fast green, see for legend figure 3).

Osteoarthritic lesions on the medial side of the femoropatellar joint was the most pronounced effect on the knee joint cartilage of mice injected with collagenase. Also

the spontaneous osteoarthritis models in STR/IN and STR/ORT mice show a preferential development of the osteoarthritic lesions on the medial side of the femoropatellar joint and the same localization is also reported for the spontaneous osteoarthritis in guinea pigs[24,25,33,37-39]. Intrinsic factors in the structure of the knee joints of these animals, or of the chondrocytes on the medial side of the femoral joint[71], might predispose to osteoarthritis in this area. A similar localization of the osteoarthritic lesions is observed in some of the surgically induced models of osteoarthritis but this might be due to the localization of the surgical intervention[18,20,21,23].

Figure 12: Spontaneous osteoarthritis in a 18 month old C57BL/10 mouse (frontal section, original magnification 40x, safranin O-fast green, see for legend figure 3).

In all three models, one of the main and first observed aspects of the alterations in the cartilage, preceding the erosive alterations, was loss of safranin O staining. After injection of papain this could be seen already after one day but in the other models depletion of stages severely affected cartilage areas in later stages, could be observed from day seven. A cellular reaction could be seen in these proteoglycan depleted cartilage in all three models, as in human osteoarthritis[1]. Some areas showed a decrease of cell number

while other adjacent areas showed chondrocyte proliferation and the presence of chondrocyte clusters. Chondrocyte proliferation was observed in the papain injected knee joints already after three days but in the knee joints injected with iodoacetate or collagenase proliferation was only observed at day 21 and later stages. Also the number of cell clusters observed was much higher after injection of papain than after injection of iodoacetate or collagenase. A high proliferative response of chondrocytes, indicating an attempt to repair the damaged matrix, is also observed in rabbits and guinea pigs after intra-articular papain injection[48,51,52,72]. Proteoglycan loss from cartilage matrix might trigger a proliferative response in chondrocytes. The proliferative response might be related to the more pronounced depletion of proteoglycans after injection of papain than after injection of the other two compounds. Iodoacetate could also have affected the chondrocyte metabolism in such a way that many of these cells loose the ability to proliferate while the changes in joint loading after collagenase injection might limit the proliferative response in the severely affected cartilage of the femorotibial joint.

In all three models we found that the remaining cells in the proteoglycan-depleted matrix and the chondrocyte clusters were often surrounded with a pericellular halo of strongly staining matrix. Similar observations have also been reported in the spontaneous osteoarthritis in other mouse strains[25,33,37-39] and in the spontaneous osteoarthritis in 18 month old C57Bl10 mice (own observations, data not shown). The presence of the strong staining around the cells might demonstrate that the chondrocytes remaining in the proteoglycan depleted matrix have a high rate of proteoglycan synthesis.

Also the glycosaminoglycan synthesis in the whole patellae shows a high rate of proteoglycan synthesis at certain stages (figure 2). After an initial inhibition on day one, an elevation of proteoglycan synthesis on day 3 and 7 after papain injection (1%) and a normal proteoglycan synthesis on day 3 and 7 after iodoacetate injection was observed. As chondrocyte death was observed after the injection of iodoacetate or papain, the high level of proteoglycan synthesis in the whole patella shows that certain chondrocytes

had an increased rate of proteoglycan synthesis. However, the rate of proteoglycan synthesis measured in the whole patella after seven days and at later stages can be affected by the formation of osteophyte cartilage at the margins of the patellae. The stimulation of proteoglycan synthesis in the patellae at day 3 and day 7 after collagenase injection might be due to the initial depletion of proteoglycans observed in the patellae on day one and day 3. At later stages, the elevated synthesis can be caused by osteophyte formation. Autoradiography studies are being carried out at the moment to solve these questions.

The osteoarthritic alterations observed after the injection of iodoacetate, papain or collagenase were not confined to changes in the articular cartilage. Osteophyte formation, a characteristic of osteoarthritis in humans and animal models, could be seen in all three models. Like the erosion of cartilage, also the formation of osteophytes was most pronounced at the margins of the femoropatellar joint after papain or iodoacetate injection and at the margins of the femorotibial joint after injection of collagenase. This demonstrates that the localization of osteophyte formation is associated with the localization of cartilage erosion in these models. The osteophytes were most times bigger on the medial side of the joint than on the lateral side of the joint. This can be related to the distribution of loading in the mouse knee joint.

Metaplastic changes were observed in the synovial tissue in all three models. This was most pronounced in the knee joints of mice injected with collagenase. The collateral ligaments on the medial side of the joints and to a lesser extent on the lateral side showed striking cartilaginous changes. Sclerosis of subchondral bone was evident 42 days after the injection of collagenase but not in the other two models. The last phenomenon might be related to the erosion of the calcified part of the cartilage which was observed in the collagenase model but not in the other two models. Similar changes, metaplasia of synovium, sclerosis of ligaments and subchondral bone, are reported for the spontaneous mouse models[25,33-35,37-39].

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CHAPTER 9

SUSCEPTIBILITY OF NORMAL AND DISEASED ARTICULAR CARTILAGE GLYCOSAMINOGLYCAN SYNTHESIS TO CHANGES IN INORGANIC SULFATE AVAILABILITY

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ABSTRACT

The effect of sulfate concentration in the medium on the glycosaminoglycan synthesis in articular cartilage of five different species was examined in relation to the physiological serum sulfate concentration in these species. Only the rate of sulfated glycosaminoglycan synthesis in human articular cartilage was sensitive to small deviations from the physiological sulfate concentration. A reduction of the sulfate concentration from 0.3 mM (physiological) to 0.2 mM resulted in a 33% reduction in glycosaminoglycan synthesis. In addition, we studied the effect of arthritic and "osteoarthritic" alterations in murine cartilage on the dependence of glycosaminoglycan synthesis on low sulfate concentrations. Arthritic and "osteoarthritic" cartilage had a similar dependence for the sulfate concentration in the medium as normal cartilage. Glycosaminoglycan synthesis in human articular cartilage appears to be very sensitive for the potential sulfate-depleting effects of drugs used in the treatment of rheumatoid arthritis and osteoarthritis.

Proteoglycans are, with collagen type II, the major structural components of articular cartilage. While collagen type II is responsible for the tensile strength, the proteoglycans are responsible for the pressure resistant properties of articular cartilage [2]. The highly sulfated glycosaminoglycans which are covalently bound to the core protein of the cartilage proteoglycans, are accountable for these pressure resistant traits. The negatively charged sulfate esters on the glycosaminoglycans are crucial for the optimal functioning of articular cartilage [2].

Inorganic sulfate is an essential substrate for the synthesis of glycosaminoglycans and the rate of synthesis of these biopolymers depends both in vivo and in vitro on the quantity of sulfate available to the chondrocytes [1, 3, 4, 10, 16]. Depletion of inorganic sulfate in serum of mice or rats by salicylate or paracetamol respectively, can lead to a diminished synthesis of sulfated glycosaminoglycans in vivo [3, 4, 16]. We studied if the physiological serum sulfate concentration in C57Bl10 mice, Wistar rats, hamsters and cows and men is at such a level that the maximal rate of glycosaminoglycan synthesis would be readily achieved in vivo and if small variations in sulfate concentration would significantly affect the in vivo synthesis rate.

As there is considerable variation in serum sulfate levels among healthy human individuals (0.08-0.58 mM, [11, 13]), the synthesis of sulfated glycosaminoglycans could also be highly variable as a result of the variation in serum sulfate levels. We wondered if glycosaminoglycan synthesis in cartilage from individuals with a low serum sulfate concentration would be less sensitive to low sulfate concentrations than individuals with higher serum sulfate concentrations. As a model of this, we investigated the serum sulfate concentration in relation to the susceptibility of glycosaminoglycan synthesis to low sulfate concentrations in several mouse strains and other animal species.

Several drugs, used in the treatment of rheumatoid arthritis and osteoarthritis for their analgesic and/or antiinflammatory properties, have the potential to reduce the sulfate

concentration in the serum of laboratory animals and humans [3, 4, 11, 16]. Depletion of serum sulfate can lead to inhibition of glycosaminoglycan synthesis in vivo [3, 4]. In addition, we have studied if arthritic cartilage, characterized by a decreased glycosaminoglycan synthesis [15], or "arthrotic" cartilage, characterized by an increased glycosaminoglycan synthesis [9, 10, 12], would have an altered sulfate dependence compared with normal cartilage.

MATERIAL AND METHODS

CARTILAGE SOURCES

Articular cartilage from ten weeks old male mice of various strains, male Wistar rats (150-200 g) and male Syrian hamsters (150-200 g) was obtained by careful dissection of the patella with surrounding tissue from the knee joints of these animals, according to the method of van den Berg et al [14]. Bovine metacarpal joints were purchased from a local slaughterhouse within 6 hours after killing of the animals. Slices of articular cartilage (3x3x3 mm) were cut and used for sulfate incorporation studies. Fresh human articular cartilage slices (3x3x3 mm), from the tibial plateau, were obtained from amputation specimens within four hours after amputation. The cartilage donors were elderly persons (range 60-75 year). All cartilage specimen were placed in sulfate free RPMI 1640 DM medium (Serva, Heidelberg, FRG) immediately after dissecting.

INDUCTION OF MURINE "ARTHRITIC" AND "ARTHROTIC" CARTILAGE

Unilateral arthritis was induced in the right knee joint of 10 weeks old male C57Bl10 mice by the intra-articular injection of (60 μg, 6 μl) zymosan in this joint. Two days after the zymosan injection the patellae were carefully dissected from both knee joints and placed in sulfate free medium. The zymosan arthritis leads to a decreased synthesis of sulfated glycosaminoglycans in the articular cartilage of the patella [15]. Articular cartilage with a stimulated synthesis of sulfated glycosaminoglycans, used as a model of "arthrotic" cartilage, was obtained by the intra-articular injection of the right knee joints of 10 weeks old male C57Bl10 mice with a 1% (w/v) papain solution (6 μl). The papain solution (type IV,

Sigma, St. Louis, MO) contained 0.03M cysteine (Sigma) to activate the papain. Three days after the papain injection the patellae from both knee joints were dissected and placed in sulfate free medium.

CULTURE OF ARTICULAR CARTILAGE

The articular cartilage specimens were washed with sulfate free RPMI 1640 DM medium for at least two hours. After the washing procedure the cartilage specimen were incubated for two hours in RPMI 1640 DM medium with various magnesiumsulfate (Merck, Darmstadt, FRG) concentrations (0.1-1.5 mM) at 37° C in a humidified 5% CO_2 atmosphere. In the medium containing less than 1.5 mM magnesiumsulfate, the deficient ions were replaced by magnesiumchloride (Merck). In pilot experiments, a sulfate concentration of 1.5 mM appeared to be an optimal concentration for glycosaminoglycan synthesis of all species studied. The RPMI medium was supplemented with 20 μCi [^{35}S]sulfate (carrier free, spec. act. 1200 Ci/mmol, Radiochemical Centre, Amersham, U.K.).

After incubation, the patellae (mouse, rat and hamster cartilage) were washed twice with physiological saline and subsequently fixed in ethanol (96%, v/v, Merck). Decalcification of the patellae with 5% formic acid (Merck) was followed by stripping of the patellar cartilage layer [5]. Patellar cartilage was digested overnight by lumasolve (Hicol, Oud-Beijerland, The Netherlands) and the amount of incorporated radiolabel was assayed by liquid scintillation analysis. As a measure of glycosaminoglycan synthesis the total sulfate incorporation per patella was calculated.

The articular cartilage slices (human and bovine cartilage) were washed twice with physiological saline to remove the free radiolabel. Thereafter, the cartilage slices were rapidly dried with a paper tissue and immediately weighed. The slices were digested by lumasolve and the quantity of incorporated radiolabel was determined by liquid scintillation analysis. The total sulfate incorporation per mg wet weight was calculated.

DETERMINATION OF SERUM SULFATE CONCENTRATION

To assay the sulfate concentration in the serum of mice,

rats and hamsters, blood samples were taken by orbita-punction under ether anesthesia. Human blood was collected from five healthy volunteers. Synovial fluid was collected from the bovine metacarpal joints and the sulfate concentration in the fluid was assayed. The sulfate concentrations in serum and synovial fluid are identical [5]. The inorganic sulfate concentration in synovial fluid and serum was determined by a modification of the benzidine method of Dogson and Spencer as recently described [5].

STATISTICAL ANALYSIS

Statistical analysis of data was performed by the two-tailed Student t-test and the Pearson correlation test. A P-value <0.05 was considered significant. We calculated for the different species the deviation of the sulfate concentration from the physiological serum sulfate concentration resulting in a significant inhibition of glycosaminoglycan synthesis. If none of the sulfate concentrations tested was in the range of the sulfate concentration ± s.d. we used the concentration just above the physiological sulfate concentration for statistical evaluation.

RESULTS

Figure 1 shows the effect of the sulfate concentration in the medium on the synthesis of sulfated glycosaminoglycans in murine patellar cartilage. Major differences in inhibition of glycosaminoglycan synthesis at low sulfate concentrations between the six mouse strains tested were not observed. A significant inhibition of sulfate incorporation was observed at sulfate concentrations below 0.5 mM for al mouse strains. In table 1 the sulfate concentration in the medium resulting in a 50% inhibition of glycosaminoglycan synthesis together with the serum sulfate concentrations in the mouse strains is presented. The serum sulfate concentration in the Balb/c strain was significantly higher than in the ICR-Br, Swiss and C57Bl10 strains. There was no correlation between the serum sulfate concentration of the various mouse strains and the susceptibility to low sulfate concentrations of cartilage glycosaminoglycan synthesis of the mouse strains. The sulfate

concentration in the serum of the mouse strains was sufficient for an optimal synthesis of glycosaminoglycans.

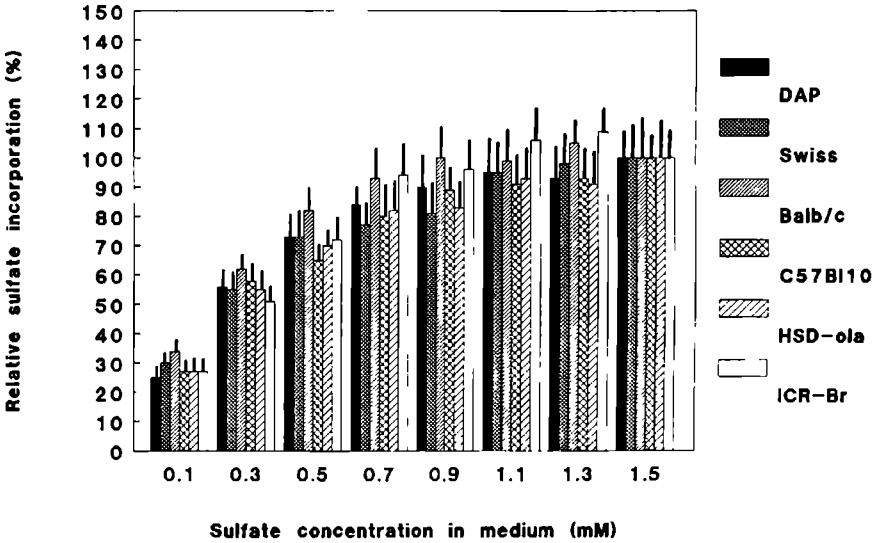

Figure 1: The effect of the sulfate concentration in the medium on the incorporation of sulfate in articular cartilage of various mouse strains. The values are expressed as a percentage of the sulfate incorporation at 1.5 mM sulfate. Each bar shows the mean ± s.d. of the incorporation of at least five patellae.

Of five different species, the physiological serum sulfate concentration and the sulfate concentration resulting in 50% inhibition of sulfated glycosaminoglycan synthesis are expressed in table 2. Figure 2 shows the sulfate dependence of glycosaminoglycan synthesis in relation to the physiological sulfate concentration in the different species. The serum sulfate concentration in human serum was significantly lower than the concentration in the serum of the other species and the physiological sulfate concentration in cows and mice was significantly higher than this level in rats and hamsters. There was no correlation between the serum sulfate concentration in the serum or synovial fluid of the species studied and the susceptibility of articular cartilage for low sulfate concentrations.

Table 1: Sulfate concentration in the medium resulting in a 50% inhibition of maximal in vitro sulfate incorporation in murine articular cartilage and the corresponding physiological sulfate concentration. The sulfate concentration of 1.5 mM is considered to be optimal under the culture conditions used and the sulfate incorporation at this concentration is set at 100%. The sulfate concentrations resulting in a 50% inhibition are expressed as the mean values (range) of at least three experiments. The values of the physiological sulfate concentration are expressed as the mean ± s.d..

Mouse strain	Sulfate concentration 50% inhibition (mM)	Sulfate concentration in serum (mM)
ICR-Br	0.25 (0.23-0.27)	0.95 ± 0.13 (n=10)
HSD-ola	0.22 (0.20-0.25)	1.15 ± 0.14 (n=10)
DAP	0.26 (0.23-0.30)	1.18 ± 0.19 (n=10)
Swiss	0.26 (0.25-0.27)	0.99 ± 0.13 (n=10)
C57Bl10	0.25 (0.22-0.29)	1.03 ± 0.09 (n=10)
Balb/c	0.22 (0.20-.025)	1.27 ± 0.14 (n=10)

We calculated for the different species the deviation of sulfate concentration in the medium from the physiological sulfate level resulting in a significant suppression of glycosaminoglycan synthesis. A change in sulfate concentration in the medium from 0.3 mM to 0.2 mM led to a significant inhibition of glycosaminoglycan synthesis (33%) in human cartilage. A decrease in sulfate concentration from 0.9 to 0.5 mM led to a significantly reduced rate of synthesis in hamsters and rats. Mouse and bovine cartilage appeared to be least sensitive to alterations in sulfate concentration. Reduction from 1.1 to 0.5 Mm sulfate resulted in a significantly diminished glycosaminoglycan synthesis in these species. Human articular cartilage was far more sensitive to changes in sulfate availability than the other species tested. We also studied the effects of a decreased glycos-aminoglycan synthesis and an enhanced glycosaminoglycan synthesis of articular cartilage on the susceptibility for low sulfate concentrations. Arthritic cartilage, with a decreased glycosaminoglycan synthesis, as well as "arthrotic" cartilage,

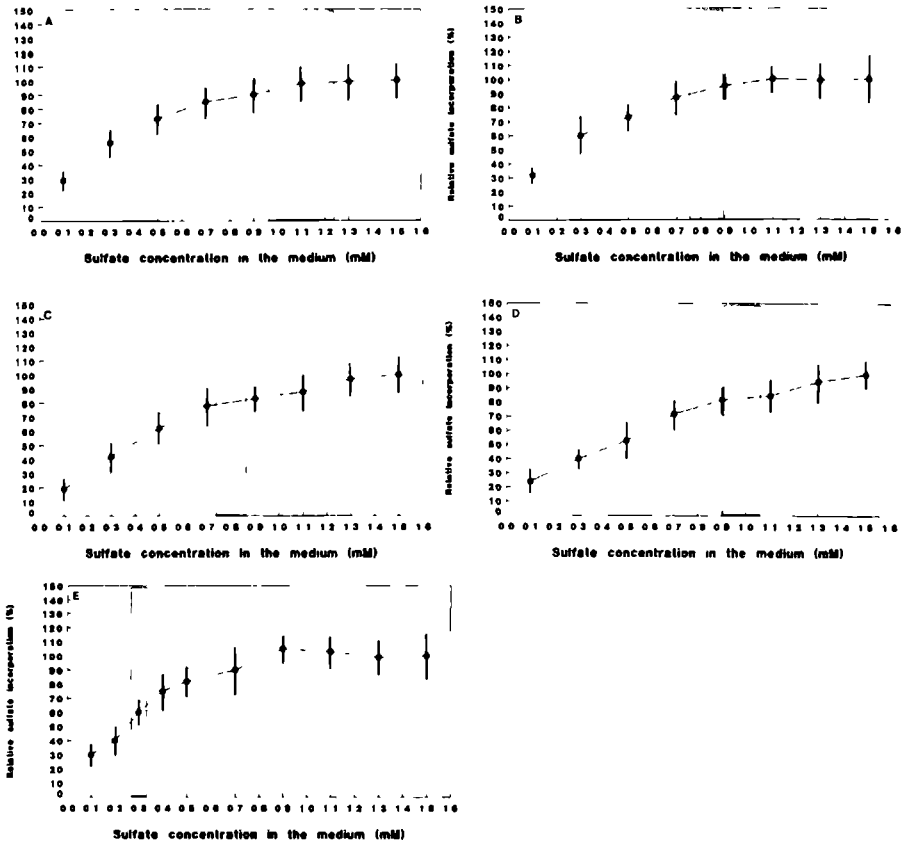

Figure 2: The effect of the sulfate concentration in the medium on the sulfate incorporation in articular cartilage of five different species. The values are expressed as a percentage of the sulfate incorporation at 1.5 mM sulfate. Each point shows the mean ± s.d. of at least five patellae or cartilage slices. The bars represent the physiological sulfate concentration ± s.d. in the corresponding species (A mouse, B rat, C hamster, D cow, E human).

with an elevated glycosaminoglycan synthesis was induced in the right knee joints of C57Bl10 mice. The incorporation of sulfate in the arthritic joints was 70% and in the "arthrotic" joints 155% of the sulfate incorporation in the contralateral control knee joints. As can be seen in figure 3, both arthritic cartilage and "arthrotic" cartilage showed no significantly different susceptibility for low sulfate concentration compared to normal cartilage.

Table 2: Sulfate concentration in the medium resulting in a 50% inhibition of maximal in vitro sulfate incorporation in articular cartilage and the corresponding physiological sulfate concentration in five different species. The mouse values are the mean values of table 1. The sulfate concentration of 1.5 mM is considered to be optimal under the culture conditions used and the sulfate incorporation at this point is set at 100%. The sulfate concentrations resulting in a 50% inhibition are expressed as the mean values (range) of at least three experiments. The values of the physiological sulfate concentration are expressed as the mean ± s.d..

Species	Sulfate concentration 50% inhibition (mM)	Sulfate concentration in serum (mM)
Mouse	0.24 (0.20-0.30)	1.10 ± 0.13 (n=60)
Hamster	0.39 (0.36-0.43)	0.80 ± 0.07 (n=10)
Rat	0.23 (0.20-0.25)	0.83 ± 0.08 (n=10)
Cow	0.49 (0.45-0.54)	0.98 ± 0.08 (n=3)
Human	0.25 (0.22-0.27)	0.30 ± 0.03 (n=5)

DISCUSSION

Inorganic sulfate is an essential substrate in the synthesis of cartilage glycosaminoglycans and this and other studies show that sulfate availability can be a rate limiting factor in the synthesis of these glycosaminoglycans [1, 3, 4, 10, 16]. The incorporation of sulfate is a reflection of the rate of synthesis of sulfated glycosaminoglycans in cartilage. The reduced incorporation of sulfate, seen at low sulfate

concentrations, is not the result of the synthesis of undersulfated glycosaminoglycans. Undersulfation of glycosaminoglycans occurs only at very low sulfate concentrations in the medium but not in the sulfate concentration range we tested in the above described experiments [17, 18].

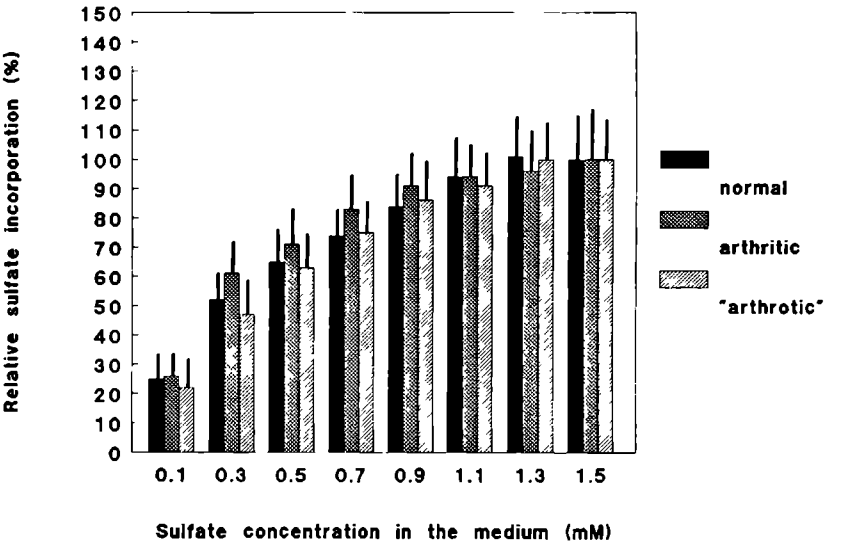

Figure 3: The effect of the sulfate concentration in the medium on the sulfate incorporation in arthritic, "arthrotic" and normal murine cartilage. The values are expressed as a percentage of the sulfate incorporation at 1.5 mM sulfate. Each bar shows the mean ± s.d. of at least five patellae. The incorporation of sulfate in the arthritic joints was 70% and in the "arthrotic" joints 155% of the sulfate incorporation in the contralateral knee joints.

Of all species studied, only the sulfate concentration in human serum was significantly suboptimal for the incorporation of sulfate in articular cartilage while the sulfate concentration in the serum of the other species was sufficient to support maximal sulfate incorporation, at least in vitro (figure 2). Moreover, already small variations in serum sulfate concentrations in humans will result in an altered rate of glycosaminoglycan synthesis in articular cartilage

while the rate of glycosaminoglycan synthesis in the other species was relatively insensitive to concentration changes. So, glycosaminoglycan synthesis in human cartilage will be very sensitive for the potential sulfate-depleting effects of drugs used in the treatment of rheumatoid arthritis and osteoarthritis [3, 4, 11, 16].

There was neither a correlation between the sulfate concentration in the serum of the mouse strains and the susceptibility of glycosaminoglycan synthesis in murine articular cartilage nor a correlation between serum sulfate concentration in the different species and the sensitivity of glycosaminoglycan synthesis for low sulfate concentrations of these species. So, species and strains with a low serum sulfate concentration do not appear to be less susceptible to lowering of the sulfate concentrations than species with a high serum sulfate concentration. When these results can be extrapolated to human individuals, the rate of glycosaminoglycan synthesis will be highly variable among human individuals as a consequence of the observed variation in serum sulfate concentrations [13]. One of the first events in the pathogenesis of osteoarthritis is an reduced content of glycosaminoglycans in the affected articular cartilage [8, 9]. Individuals with a low serum sulfate concentration might have a lower capacity of glycosaminoglycan synthesis and as a result of this also a more limited potential of matrix repair than individuals with a high serum sulfate concentration. As a consequence of this, individuals with a low serum sulfate concentration might be more vulnerable to the development of osteoarthritis than those with a higher serum sulfate level.

The dependence of glycosaminoglycan synthesis on the sulfate concentration in the medium was similar for arthritic, "arthrotic" and normal cartilage. Arthritic and "arthrotic" cartilage was not more or less vulnerable to low sulfate concentrations than normal cartilage. Variation in serum sulfate concentrations, potentially induced by antirheumatic medication [3, 4, 6, 16], will result in similar changes in the rate of glycosaminoglycan synthesis in normal and arthritic or "arthrotic" cartilage.

The results of this study show that already a slight alteration in sulfate availability will result in a

significant change in glycosaminoglycan synthesis in human cartilage, this in contrast to the other species tested. As a consequence of this the rate of glycosaminoglycan synthesis in humans will be sensitive to drug-induced depletion of serum sulfate [11] and this sensitivity might play a role in the susceptibility of certain individuals to osteoarthritis.

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CHAPTER 10

SUMMARY AND FINAL REMARKS

Articular cartilage is a specialized form of connective tissue consisting of chondrocytes surrounded by an extracellular matrix of mainly giant macromolecules synthesized by these chondrocytes. The extracellular matrix macromolecules, predominantly proteoglycans and collagen type II, are responsible for the biomechanical and biochemical properties making articular cartilage unique in its function as gliding surface and shock absorber for the diarthrodal joint. The proteoglycan molecules in the extracellular matrix are responsible for the resiliency and water binding capacity of articular cartilage. The sulfate esters of the glycosaminoglycan side chains of proteoglycans are essential for these functions.
Several drugs used in the treatment of osteoarthritis and rheumatoid arthritis have the potential of serum sulfate depletion (chapter 2) and can theoretically lead by this way to:

1 A decreased synthesis of glycosaminoglycans in articular cartilage.
2 The synthesis of undersulfated cartilage glycosaminoglycans.
3 A combination of both 1 and 2.

To test this hypothesis we have incubated murine patellar cartilage in incubation medium with various sulfate concentrations (chapter 3 and 4). A medium sulfate concentration below 0.5 mM significantly inhibited the glycosaminoglycan synthesis as measured by incorporation of sulfate. The incorporation of glucosamine was unaltered at low sulfate concentrations indicating the synthesis of undersulfated glycosaminoglycans. Characterization of glycosaminoglycans synthesized at a sulfate concentration of 0.1 mM (physiological sulfate concentration), by anion exchange chromatography and HPLC disaccharide analysis, could not confirm the synthesis of undersulfated glycosaminoglycans (chapter 3). The synthesis of aberrant glycosaminoglycans only occurred at very low sulfate concentrations (10 nM, in our experiments), irrelevant for the in vivo situation. The observation of a unaltered incorporation of glycosaminoglycans

at reduced sulfate concentrations might be the result of an increase in intracellular specific activity of glucosamine under conditions favouring a reduced glycosaminoglycan synthesis.

We also tested the effect of serum sulfate depletion on articular cartilage synthesis in vivo (chapter 5 and 6). Depletion of serum sulfate was induced in Wistar rats by the oral administration of paracetamol. Administration of 190 mg/Kg paracetamol to these rats led within five hours to a reduction of inorganic serum sulfate from 0.8 mM to 0.1 mM (chapter 5). The reduced availability of sulfate resulted in a 35% inhibition of glycosaminoglycan synthesis in the patellar cartilage. The biochemical characteristics of the glycosaminoglycans synthesized under these conditions were similar with those of glycosaminoglycans produced at physiological sulfate concentrations.

Long-term treatment of Wistar rats with paracetamol resulted during the first three weeks in a significantly reduced concentration of serum sulfate (chapter 6). From four weeks up to the end of the experiments (nine weeks) the serum sulfate level in the paracetamol-treated rats was however not significantly different from this level in control rats. An adaptation of the Wistar rats to the paracetamol-treatment appears to overcome the sulfate depletion within several weeks. Similar results are reported for the human situation. Although short-term administration of paracetamol leads to significantly reduced serum sulfate levels in human volunteers, long-term administration of paracetamol to patients even elevates the serum sulfate levels above normal (1).

The glycosaminoglycan content of patellar cartilage was only significantly lower in the paracetamol-treated rats than in the control rats after three and four weeks of treatment. After two weeks or nine weeks treatment the patellar glycosaminoglycan content of the paracetamol-treated rats was comparable with that content in control rats. A two week treatment was probably to limited to induce a significantly reduced patellar glycosaminoglycan content while after nine weeks the patellar cartilage has been able to recover from the reduced glycosaminoglycan content.

In addition to the routinely used murine arthritis models, three murine osteoarthritis models were developed (chapter 7 and 8). A intra-articular injection of murine knee joints with collagenase led to joint instability eventually resulting in osteoarthritis (chapter 7). Injection of papain or iodo-acetate led to osteoarthritic lesions in the knee joints by interference with cartilage metabolism (chapter 8).
Both articular cartilage with an inhibited glycosaminoglycan synthesis (arthritic) or an elevated glycosaminoglycan synthesis (osteoarthritic) appeared to have a similar dependence on sulfate availability as normal articular cartilage (chapter 9).
The susceptibility of glycosaminoglycan synthesis in articular cartilage of five different species to deviations in inorganic sulfate concentrations from the physiological serum sulfate concentration in the corresponding species was studied (chapter 9). Human articular cartilage glycosaminoglycan synthesis appeared to be more sensitive to small deviations from the normal serum sulfate level (0.3 mM) than the glycosamino-glycan synthesis in the other species tested.

Finally, the above described studies demonstrate that the glycosaminoglycan synthesis in human articular cartilage will already be inhibited by a slight reduction in serum sulfate concentration. As many drugs currently used in the treatment of patients suffering from osteoarthritis and rheumatoid arthritis have the potential, at least in laboratory animals, to reduce inorganic serum sulfate levels, these drugs might interfere with glycosaminoglycan synthesis. However, from our data it appears unlikely that the synthesis of aberrant glycosaminoglycans will be induced by drug-induced sulfate depletion. Moreover, a drug well known for its serum sulfate depleting effects in humans and animals after short-dosing, paracetamol, had no significant effects on serum sulfate during long-term treatment both in rats and humans (1).

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van het proefschrift, getiteld

EFFECT VAN SULFAATVERLAGENDE FARMACA OP HET KRAAKBEENMETABOLISME

Articulair kraakbeen is een gespecialiseerd type bindweefsel dat het contactvlak vormt tussen articulerende gewrichten. Het bestaat uit een extracellulaire matrix van reusachtige macromolekulen, voornamelijk collageen type II en proteoglycanen, spaarzaam bezaaid met kraakbeencellen (chondrocyten) die de extracellulaire matrix produceren. De extracellulaire matrixmolekulen zijn verantwoordelijk voor de unieke schokabsorberende en wrijvingreducerende eigenschappen van articulair kraakbeen. De negatief geladen sulfaatesters op de glycosaminoglycaan zijketens van de proteoglycaanmolekulen zijn essentieel voor de reversibele vervormbaarheid (veerkracht) van het kraakbeen. Verstoring van de sulfatering of de synthesesnelheid van kraakbeen glycosaminoglycanen kan derhalve leiden tot verlies van de funktionele en anatomische integriteit van het gewrichtskraakbeen.
Verschillende farmaca gebruikt bij de behandeling van arthrose en/of reumatoide arthritis patienten (b.v. salicylaten, tiaprofeenzuur, chloroquine diphosphaat) kunnen potentieel de sulfaatconcentratie in het serum verlagen (op zijn minst in muizen en ratten, hoofdstuk 2 en 5) en via deze weg aanleiding geven tot:

1 Een verminderde synthese van glycosaminoglycanen in articulair kraakbeen.
2 De synthese van ondergesulfateerde kraakbeen glycosaminoglycanen.
3 Een combinatie van 1 en 2

Deze hypothesen hebben we getest door muizeknieschijfjes in vitro (in glas) te incuberen in aanwezigheid van verschillende sulfaatconcentraties in het kweekmedium (hoofdstuk 3 en 4). Een sulfaatconcentratie in het medium beneden 0,5 mM leidde tot een verminderde inbouw van sulfaat in de kraakbeenglycosaminoglycanen. De inbouw van glucosamine (een andere bouwsteen van glycosaminoglycanen) bleef echter constant. Dit duidde op de synthese van ondergesulfateerde glycosaminoglycanen. Echter analyse van de glycosaminoglycanen gesynthetiseerd bij 1,0 mM sulfaat (physiologisch voor de muis) en 0,1 mM sulfaat kon de synthese van ondergesulfateerde glycosaminglycanen bij een verlaagd sulfaataanbod niet bevestigen. Alleen bij een zeer lage sulfaatconcentratie (10 nM) kon de synthese van ondergesulfateerde glycosaminoglycanen worden aangetoond.

De verminderde sulfaatinbouw is een gevolg van de verminderde produktie van glycosaminoglycanen bij een laag sulfaataanbod. Het feit dat de inbouw van glucosamine niet afneemt bij een verminderde glycosaminoglycaan synthese wordt waarschijnlijk veroorzaakt door veranderingen in de intracellulaire specifieke activiteit van glucosamine onder condities met een geremde glycosaminoglycaan synthese.
Naast deze in vitro experimenten is ook het effect van sulfaat verlaging in vivo op glycosaminoglycaan synthese bestudeerd (hoofdstuk 5 en 6). Orale toediening van 190 mg/Kg paracetamol aan Wistar ratten leidde binnen vijf uur tot een verlaging van de sulfaatconcentratie in het serum van 0,8 mM tot 0,1 mM (hoofdstuk 5). Deze verlaging van de serumsulfaat-concentratie resulteerde in een 35% remming van de glycosaminoglycaan synthese in het kraakbeen van de ratteknieschijfjes. Het verminderde sulfaataanbod had geen effect op de biochemische eigenschappen van de gesynthetiseerde glycosaminoglycanen.
Het langdurig toedienen van paracetamol aan Wistar Ratten resulteerde gedurende de eerste drie weken in een significant verlaagde serum sulfaatconcentratie (hoofdstuk 6). Na deze eerste drie weken had paracetamol minder effect dan verwacht en een significante verlaging van de serum sulfaatconcentratie werd niet meer waargenomen. De chronische paracetamol behandeling had geen aantoonbaar effect op de nier- en leverfunctie. Het glycosaminoglycaangehalte was na 3 en 4 weken behandeling met paracetamol significant lager in het kraakbeen van het knieschijfje van de behandelde groep dan in het kraakbeen van de controle groep. Na 2 en 9 weken waren er geen significante verschillen aantoonbaar. Twee weken paracetamol behandeling was waarschijnlijk te kort om een significant verschil te bewerkstelligen terwijl na negen weken het kraakbeen weer de kans had gekregen voor herstel van het glycosaminoglycaangehalte gedurende de fase dat paracetamol geen effect had op de serum sulfaatconcentratie.
In alle bovenbeschreven experimenten is het effect van een laag sulfaataanbod op normaal kraakbeen bestudeerd. Om het effect van een laag sulfaataanbod tevens op artritisch en artrotisch kraakbeen te kunnen bestuderen is naast de in ons laboratorium routinematig gebruikte gewrichtsontstekingsmodellen in muizen een aantal arthrose modellen in muizen ontwikkeld (hoofdstuk 7 en 8). Een éénmalige intra-articulaire injectie met collagenase in het

muizekniegewricht leidt tot gewrichtsinstabiliteit en dit resulteert uiteindelijk in osteoarthrotische veranderingen in het kniegewricht (hoofdstuk 7). Een injectie met papaine of joodacetaat in het kniegewricht leidt tot osteoarthrotische veranderingen in het gewricht door middel van interferentie met het kraakbeenmetabolisme (hoofdstuk 8).
Zowel muizekraakbeen met een geremde glycosaminoglycaan synthese (arthritisch) als muizekraakbeen met een gestimuleerde glycosaminoglycaan synthese (arthrotisch) bleek even gevoelig te zijn voor een laag sulfaataanbod als normaal kraakbeen (hoofdstuk 9). De glycosaminoglycaan synthese in humaan kraakbeen bleek gevoeliger te zijn voor kleine afwijkingen in het sulfaataanbod (ten opzichte van de fysiologische serum sulfaatconcentratie) dan de glycosaminoglycaan synthese in vier andere bestudeerde soorten (hoofdstuk 9).

Uit het bovenbeschreven onderzoek blijkt dat de synthese van glycosaminoglycanen in het kraakbeen van de mens al kan worden geremd bij een geringe verlaging van het sulfaataanbod. Verscheidene farmaca momenteel in gebruik bij de behandeling van patienten met gewrichtsziekten, blijken op zijn minst in proefdieren aanleiding te kunnen geven tot een verlaagde sulfaatconcentratie in het serum van deze dieren en kunnen via deze weg mogelijk aanleiding leiden tot een verminderde synthese van glycosaminoglycanen in het kraakbeen. Echter, onze studies wijzen erop dat de verlaging van de serum sulfaatconcentratie door middel van farmaca niet zal leiden tot de synthese van kwalitatief inferieure glycosaminoglycanen. Tevens blijkt dat het medicament paracetamol, bekend om zijn sulfaatverlagende eigenschappen zowel bij proefdieren als bij de mens na een éénmalige dosis, geen significant effect heeft op de serum sulfaatconcentratie bij langdurige toediening.

Allen die op enigerlei wijze hebben bijdragen aan het tot stand komen van dit proefschrift wil ik bedanken voor hun inzet.

Naast al deze mensen gaat mijn bijzondere dank uit naar Elly Vitters voor de prettige samenwerking en haar belangrijke aandeel bij het uitvoeren van de experimenten. Elly, je zelfstandige werkwijze en je volledige inzet hebben een belangrijke bijdrage geleverd aan het tot stand komen van dit proefschrift.

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Ben de Vries ben ik erkentelijk voor zijn introductie in de sulfaat- en kraakbeenproblematiek.

Verder wil ik Onno, John, Henk, Peter, Liduine, Fons, Maries, Peter, Mieke, Leo, Anita, Monique, Wim, Lex, Joost, Marianne, Marike en alle andere medewerkers en exmedewerkers van het research laboratorium Reumatologie bedanken voor de uitstekende werksfeer.

De auteur van dit proefschrift, Peter van der Kraan, werd op 6 november 1959 geboren te Schiedam. Na het eindexamen Atheneum B aan het Maaslandcollege te Oss in 1978, begon hij de studie biologie aan de Katholieke Universiteit Nijmegen. Het behalen van het kandidaatsexamen in 1981 werd gevolgd door een oecologische hoofdvakstage op de afdeling Zoologie III (Prof. Denucee). De onderwerpen van de beide bijvakken waren respectievelijk "Effecten van nucleosiden op leukemische en niet-leukemische cellijnen" (Anthropogenetisch Instituut, Prof. Geerts) en "Inductie en suppressie van 'delayed type hypersensitivity' bij muizen" (Afd. Nefrologie, Prof. Koenen). Na het behalen van het doctoraalexamen heeft de auteur van juli 1984 tot maart 1986 onderzoek verricht binnen het kader van een door het Koningin Wilhelmina Fonds gesubsidieerd leukemieproject (Kinder Oncologisch Centrum, Academisch Ziekenhuis St. Radboud, Nijmegen). In 1986 en 1988 werden respectievelijk de diploma's NIMA A en NIMA B (Marketing Manager) behaald. Sinds maart 1986 is de auteur verbonden aan de afdeling Reumatologie van het St. Radboud Ziekenhuis, gedurende deze periode heeft de auteur gewerkt aan het door de Nederlandse Vereniging voor Reumabestrijding gesubsidieerde project "Effect van sulfaatdepletie op kraakbeen" wat zijn weerslag heeft gevonden in dit proefschrift.

Peter van der Kraan is gehuwd met Ellen van de Wijgert, samen hebben ze twee zoons, Maarten en Thomas.

STELLINGEN

I

Verlaging van de sulfaatconcentratie in het serum van ratten leidt tot een verminderde synthese van glycosaminoglycanen in articulair kraakbeen.
Dit proefschrift

II

Synthese van biochemisch afwijkende glycosaminoglycanen in articulair kraakbeen zal nooit het gevolg zijn van een verlaagd sulfaataanbod in vivo.
Dit proefschrift

III

Wanneer het effect van farmaca op de synthese van glycosaminoglycanen in vivo wordt gemeten met behulp van de inbouw van radioactief sulfaat moet het effect van deze farmaca op de sulfaathomeostase worden verdisconteerd.
Dit proefschrift

IV

In tegenstelling tot een eenmalige dosis leidt de langdurige toediening van paracetamol niet tot een verlaagde serum sulfaatconcentratie.
(S. Hendrix-Treacy et al., Eur. J. Clin. Pharmacol. 30:273-278, 1986)
Dit proefschrift

V

De glycosaminoglycaansynthese in humaan articulair kraakbeen is uitermate gevoelig voor veranderingen in de serum sulfaatconcentratie rond de fysiologische concentratie.
Dit proefschrift

VI

Een eenmalige intra-articulaire injectie van collagenase resulteert in arthrotische veranderingen in het muizekniegewricht ten gevolge van het ontstaan van gewrichtsinstabiliteit.
Dit proefschrift

VII

De observatie dat transforming growth factor β de glycosaminoglycaan-synthese van geïsoleerde runderchondrocyten de ene keer remt en de andere keer stimuleert toont aan dat opheldering van de regulerende rol van groeifactoren op het celmetabolisme nog een lange weg te gaan heeft.
(eigen waarneming)

VIII

De verlaagde proteoglycaanconcentratie in arthrotisch kraakbeen is niet alleen het gevolg van de proteolytische afbraak van proteoglycanen maar tevens van een verminderde hyaluronzuurconcentratie in arthrotisch kraakbeen.
(D.H. Manicourt & J.C. Pita, Arthritis Rheum. 31:538-544, 1988)

IX

Dat een hogere concentratie van een bepaalde werkzame stof niet altijd meer effect heeft dan een lagere concentratie is gemeengoed onder biochemici, maar dat meer soms leidt tot minder is voor velen een te grote gedachtensprong.
(P.M. van der Kraan et al., Biochim. Biophys. Acta 927:213-221, 1987)

X

Een intense communicatie tussen de leden van een organisatie is niet alleen noodzakelijk voor uitmuntendheid van innovatieprocessen binnen een industriele setting maar ook binnen een onderzoekslaboratorium.
(Naar: T.J. Peters & R.H. Waterman, "In search of excellence", Harper & Row, New York, 1982)

XI

De vergrijzing van de Nederlandse bevolking maakt het noodzakelijk om het onderzoek naar arthrose zodanig te stimuleren dat het tempo van "voetje voor voetje" verandert in een "gestrekte draf".

XII

Het feit dat een eeneiige tweeling door velen beschouwd wordt als een bijzondere bezienswaardigheid is niet bevorderlijk voor de gemoedsrust van de ouders.

(eigen waarneming)

XIII

De onder het grote publiek heersende opvatting dat het doel van karate voornamelijk ligt in het vernielen van bouwmateriaal wordt in de hand gewerkt door de ledenwervende activiteiten van commerciele sportscholen.

XIV

Het is niet alleen bij verzekeringen maar ook bij dissertaties van belang om "de kleine lettertjes" te lezen.

Stellingen bij het proefschrift:

THE EFFECT OF SULFATE DEPLETING DRUGS ON CARTILAGE METABOLISM

Peter van der Kraan
30 november 1989